



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> :  C12N 15/85, 15/57	A1	(11) International Publication Number: <b>WO 90/05188</b>  (43) International Publication Date: 17 May 1990 (17.05.90)
<p>(21) International Application Number: PCT/GB89/01343</p> <p>(22) International Filing Date: 13 November 1989 (13.11.89)</p> <p>(30) Priority data: 8826446.0 11 November 1988 (11.11.88) GB</p> <p>(71) Applicant (for all designated States except US): PHARMA-CEUTICAL PROTEINS LIMITED [GB/GB]; Orchard Brae House, 30 Queensferry Road, Edinburgh EH4 2HG (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : ARCHIBALD, Alan, Langskill [GB/GB]; 70 Silverknowes View, Edinburgh EH4 5PS (GB). CLARK, Anthony, John [GB/GB]; 29 Broomieknowe, Lasswade, Midlothian EH18 1LN (GB). HARRIS, Stephen [GB/GB]; 3 Leamington Road, Edinburgh EH3 9PD (GB). McCLENAGHAN, Margaret [GB/GB]; 8 Livingstone Place, Edinburgh EH9 1PA (GB). SIMONS, John, Paul [GB/GB]; 94 Warrender Park Road, Edinburgh EH9 1ET (GB). WHITE LAW, Christopher, Bruce, Alexander [GB/GB];</p>	<p>43 Viewforth, Edinburgh EH10 4LA (GB).</p> <p>(74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn &amp; Strode, 30 John Street, London WC1N 2DD (GB).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU, US.</p> <p><b>Published</b> <i>With international search report.</i></p>	
<p>(54) Title: A GENETIC CONSTRUCT OF WHICH PROTEIN-CODING DNA COMPRISES INTRONS AND IS DESIGNED FOR PROTEIN PRODUCTION IN TRANSGENIC ANIMALS</p>		
<p>(57) Abstract</p> <p>Proteinaceous products can be produced by transgenic animals having genetic constructs integrated into their genome. The construct comprises a 5'-flanking sequence from a mammalian milk protein gene (such as beta-lactoglobulin) and DNA coding for a heterologous protein other than the milk protein (for example a serin protease such as alpha<sub>1</sub>-antitrypsin or a blood factor such as Factor VIII or IX). The protein-coding DNA comprises at least one, but not all, of the introns naturally occurring in a gene coding for the heterologous protein. The 5'-flanking sequence is sufficient to drive expression of the heterologous protein.</p>		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

1 A genetic construct of which protein coding DNA comprises  
2 introns and is designed for protein production in trans-  
genic animals.

3 This invention relates to the production of  
4 peptide-containing molecules.

5  
6 Recombinant DNA technology has been used increasingly  
7 over the past decade for the production of commercially  
8 important biological materials. To this end, the DNA  
9 sequences encoding a variety of medically important  
10 human proteins have been cloned. These include  
11 insulin, plasminogen activator,  $\alpha_1$ -antitrypsin and  
12 coagulation factors VIII and IX. At present, even with  
13 the emergent recombinant DNA techniques, these proteins  
14 are usually purified from blood and tissue, an  
15 expensive and time consuming process which may carry  
16 the risk of transmitting infectious agents such as  
17 those causing AIDS and hepatitis.

18  
19 Although the expression of DNA sequences in bacteria to  
20 produce the desired medically important protein looks  
21 an attractive proposition, in practice the bacteria  
22 often prove unsatisfactory as hosts because in the  
23 bacterial cell foreign proteins are unstable and are  
24 not processed correctly.

25  
26 Recognising this problem, the expression of cloned  
27 genes in mammalian tissue culture has been attempted  
28 and has in some instances proved a viable strategy.  
29 However batch fermentation of animal cells is an  
30 expensive and technically demanding process.

31  
32 There is therefore a need for a high yield, low cost  
33 process for the production of biological substances

1 al and to insert into mammalian genomes transgenes  
2 based on natural foreign genes as opposed to foreign  
3 cDNA. Unfortunately, this approach is itself  
4 problematical. First, as mentioned above, natural  
5 genes having introns will inevitably be larger than the  
6 cDNA coding for the product of the gene. This is  
7 simply because the introns are removed from the primary  
8 transcription product before export from the nucleus as  
9 mRNA. It is technically difficult to handle large  
10 genomic DNA. Approximately 20 kb, for example,  
11 constitutes the maximum possible cloning size for  
12 lambda-phage. The use of other vectors such as  
13 cosmids, may increase the handleable size up to 40 kb,  
14 but there is then a greater chance of instability. It  
15 should be noted that eukaryotic DNA contains repeated  
16 DNA sequence elements that can contribute to  
17 instability. The larger the piece of DNA the greater  
18 the chance that two or more of these elements will  
19 occur, and this may promote instability.

20  
21 Secondly, even if it is technically possible to  
22 manipulate large fragments of genomic DNA, the longer  
23 the length of manipulated DNA, the greater chance that  
24 restriction sites occur more than once, thereby making  
25 manipulation more difficult. This is especially so  
26 given the fact that in most transgenic techniques, the  
27 DNA to be inserted into the mammalian genome will often  
28 be isolated from prokaryotic vector sequences (because  
29 the DNA will have been manipulated in a prokaryotic  
30 vector, for choice). The prokaryotic vector sequences  
31 usually have to be removed, because they tend to  
32 inhibit expression. So the longer the piece of DNA,  
33 the more difficult it is to find a restriction enzyme  
34 which will not cleave it internally.

1 To illustrate this problem,  $\alpha_1$ -antitrypsin, Factor  
2 IX and Factor VIII will briefly be considered.  $\alpha_1$ -  
3 antitrypsin (AAT) comprises 394 amino acids as a mature  
4 peptide. It is initially expressed as a 418 amino acid  
5 pre-protein. The mRNA coding for the pre-protein is  
6 1.4 kb long, and this corresponds approximately to the  
7 length of the cDNA coding for AAT used in the present  
8 application (approximately 1.3 kb). The structural  
9 gene (liver version, Perlino et al, The EMBO Journal  
10 Volume 6 p.2767-2771 (1987)) coding for AAT contains 4  
11 introns and is 10.2 kb long.

12  
13 Factor IX (FIX) is initially expressed as a 415 amino  
14 acid preprotein. The mRNA is 2.8 kb long, and the cDNA  
15 that was used in WO-A-8800239 to build FIX constructs  
16 was 1.57 kb long. The structural gene is approximately  
17 34 kb long and comprises 7 introns.

18  
19 Factor VIII (FVIII) is expressed as a 2,351 amino acid  
20 preprotein, which is trimmed to a mature protein of  
21 2,332 amino acids. The mRNA is 9.0 kb in length,  
22 whereas the structural gene is 185 kb long.

23  
24 It would therefore be desirable to improve upon the  
25 yields and reliability of transgenic techniques  
26 obtained when using constructs based on cDNA, but  
27 without running into the size difficulties associated  
28 with the natural gene together with all its introns.

29  
30 It has now been discovered that high yields can be  
31 obtained using constructs comprising some but not all,  
32 of the naturally occurring introns in a gene.

33

1 According to a first aspect of the present invention,  
2 there is provided a genetic construct comprising a 5'  
3 flanking sequence from a mammalian milk protein gene  
4 and DNA coding for a heterologous protein other than  
5 the milk protein, wherein the protein-coding DNA  
6 comprises at least one, but not all, of the introns  
7 naturally occurring in a gene coding for the  
8 heterologous protein and wherein the 5'-flanking  
9 sequence is sufficient to drive expression of the  
10 heterologous protein.

11

12 The milk protein gene may be the gene for whey acid  
13 protein, alpha-lactalbumin or a casein, but the  
14 beta-lactoglobulin gene is particularly preferred.

15

16 In this specification the term "intron" includes the  
17 whole of any natural intron or part thereof.

18

19 The construct will generally be suitable for use in  
20 expressing the heterologous protein in a transgenic  
21 animal. Expression may take place in a secretory gland  
22 such as the salivary gland or the mammary gland. The  
23 mammary gland is preferred.

24

25 The species of animals selected for expression is not  
26 particularly critical, and will be selected by those  
27 skilled in the art to be suitable for their needs.  
28 Clearly, if secretion in the mammary gland is the  
29 primary goal, as is the case with preferred embodiments  
30 of the invention, it is essential to use mammals.  
31 Suitable laboratory mammals for experimental ease of  
32 manipulation include mice and rats. Larger yields may  
33 be had from domestic farm animals such as cows, pigs,

1 goats and sheep. Intermediate between laboratory  
2 animals and farm animals are such animals as rabbits,  
3 which could be suitable producer animals for certain  
4 proteins.

5  
6 The 5' flanking sequence will generally include the  
7 milk protein, e.g. beta-lactoglobulin (BLG),  
8 transcription start site. For BLG it is preferred that  
9 about 800 base pairs (for example 799 base pairs)  
10 upstream of the BLG transcription start site be  
11 included. In particularly preferred embodiments, at  
12 least 4.2 kilobase pairs upstream be included.

13  
14 The DNA coding for the protein other than BLG ("the  
15 heterologous protein") may code for any desired protein  
16 of interest. One particularly preferred category of  
17 proteins of interest are plasma proteins. Important  
18 plasma proteins include serine protease inhibitors,  
19 which is to say members of the SERPIN family. An  
20 example of such a protein is  $\alpha_1$ -antitrypsin. Other  
21 serine protease inhibitors may also be coded for.  
22 Other plasma proteins apart from serine protease  
23 inhibitors include the blood factors, particularly  
24 Factor VIII and Factor IX.

25  
26 Proteins of interest also include proteins having a  
27 degree of homology (for example at least 90%) with the  
28 plasma proteins described above. Examples include  
29 oxidation-resistant mutants and other analogues of  
30 serine protease inhibitors such as AAT. These  
31 analogues include novel protease inhibitors produced by  
32 modification of the active site of  $\alpha_1$ -antitrypsin.  
33 For example, if the Met-358 of AAT is modified to Val,

1 this replacement of an oxidation-sensitive residue at  
2 the active centre with an inert valine renders the  
3 molecule resistant to oxidative inactivation.  
4 Alternatively, if the Met-358 residue is modified to  
5 Arg, the molecule no longer inhibits elastase, but is  
6 an efficient heparin-independent thrombin inhibitor  
7 (that is to say, it now functions like anti-thrombin  
8 III).

9  
10 The protein-coding DNA has a partial complement of  
11 natural introns or parts thereof. It is preferred in  
12 some embodiments that all but one be present. For  
13 example, the first intron may be missing but it is also  
14 possible that other introns may be missing. In other  
15 embodiments of the invention, more than one is missing,  
16 but there must be at least one intron present in the  
17 protein-coding DNA. In certain embodiments it is  
18 preferred that only one intron be present.

19  
20 Suitable 3'-sequences may be present. It may not be  
21 essential for such sequences to be present, however,  
22 particularly if the protein-coding DNA of interest  
23 comprises its own polyadenylation signal sequence.  
24 However, it may be necessary or convenient in some  
25 embodiments of the invention to provide 3'-sequences  
26 and 3'-sequences of BLG will be those of choice.  
27 3'-sequences are not however limited to those derived  
28 from the BLG gene.

29  
30 Appropriate signal and/or secretory sequence(s) may be  
31 present if necessary or desirable.

32  
33



1 According to a second aspect of the invention, there is  
2 provided a method for producing a substance comprising  
3 a polypeptide, the method comprising introducing a DNA  
4 construct as described above into the genome of an  
5 animal in such a way that the protein-coding DNA is  
6 expressed in a secretory gland of the animal.

7  
8 The animal may be a mammal, expression may take place  
9 in the mammary gland, for preference. The construct  
10 may be inserted into a female mammal, or into a male  
11 mammal from which female mammals carrying the construct  
12 as a transgene can be bred.

13  
14 Preferred aspects of the method are as described in  
15 WO-A-8800239.

16  
17 According to a third aspect of the invention, there is  
18 provided a vector comprising a genetic construct as  
19 described above. The vector may be a plasmid, phage,  
20 cosmid or other vector type, for example derived from  
21 yeast.

22  
23 According to a fourth aspect of the invention, there is  
24 provided a cell containing a vector as described above.  
25 The cell may be prokaryotic or eukaryotic. If  
26 prokaryotic, the cell may be bacterial, for example E.  
27 coli. If eukaryotic, the cell may be a yeast cell or  
28 an insect cell.

29  
30 According to a fifth aspect of the invention, there is  
31 provided a mammalian or other animal cell comprising a  
32 construct as described above.

33

1 According to a sixth aspect of the invention, there is  
2 provided a transgenic mammal or other animal comprising  
3 a genetic construct as described above integrated into  
4 its genome. It is particularly preferred that the  
5 transgenic animal transmits the construct to its  
6 progeny, thereby enabling the production of at least  
7 one subsequent generation of producer animals.

8  
9 The invention will now be illustrated by a number of  
10 examples. The examples refer to the accompanying  
11 drawings, in which:

12  
13 FIGURES 1 to 10 show schematically one strategy used  
14 for elaborating fusion genes comprising DNA sequence  
15 elements from ovine beta-lactoglobulin and the gene(s)  
16 of interest, in this case  $\alpha_1$ -antitrypsin, to be  
17 expressed in the mammary gland of a mammal;

18  
19 FIGURE 11 shows a Northern blot giving the results of  
20 Example 2;

21  
22 FIGURE 12 shows an RNase protection gel, referred to in  
23 Example 2;

24  
25 FIGURE 13 shows an Immuno blot of diluted milk samples  
26 from transgenic and normal mice, referred to in Example  
27 2;

28  
29 FIGURE 14 shows a Western blot of milk whey samples  
30 from normal and two transgenic sheep (Example 3);

31  
32 FIGURE 15 shows Western blots of TCA-precipitated whey  
33 samples from normal and transgenic mice (Example 3);

1 FIGURES 16a, 16b and 17 to 20 show schematically the  
2 strategy used for elaborating a further strategy used  
3 for elaborating fusion genes comprising DNA sequence  
4 elements from ovine beta-lactoglobulin and the gene(s)  
5 of interest, in this case Factor IX, to be expressed in  
6 the mammary gland of a mammal.

7  
8 EXAMPLE 1

9  
10 General

11  
12 Where not specifically detailed, recombinant DNA and  
13 molecular biological procedures were after Maniatis et  
14 al ("Molecular Cloning" Cold Spring Harbor (1982))  
15 "Recombinant DNA" Methods in Enzymology Volume 68,  
16 (edited by R. Wu), Academic Press (1979); "Recombinant  
17 DNA part B" Methods in Enzymology Volume 100, (Wu,  
18 Grossman and Moldgave, Eds), Academic Press (1983);  
19 "Recombinant DNA part C" Methods in Enzymology Volume  
20 101, (Wu, Grossman and Moldgave, Eds), Academic Press  
21 (1983); and "Guide to Molecular Cloning Techniques",  
22 Methods in Enzymology Volume 152 (edited by S.L. Berger  
23 & A.R. Kimmel), Academic Press (1987). Unless  
24 specifically stated, all chemicals were purchased from  
25 BDH Chemicals Ltd, Poole, Dorset, England or the Sigma  
26 Chemical Company, Poole, Dorset, England. Unless  
27 specifically stated all DNA modifying enzymes and  
28 restriction endonucleases were purchased from BCL,  
29 Boehringer Mannheim House, Bell Lane, Lewes, East  
30 Sussex BN7 1LG, UK.

31  
32 [Abbreviations: bp = base pairs; kb = kilobase pairs,  
33 AAT = alpha1-antitrypsin; BLG = beta-lactoglobulin;

1   FIX = factor IX; E. coli = Escherichia coli; dNTPs =  
2   deoxyribonucleotide triphosphates; restriction  
3   endonucleases are abbreviated thus e.g. BamHI; the  
4   addition of -O after a site for a restriction  
5   endonuclease e.g. PvuII-O indicates that the  
6   recognition site has been destroyed]

7  
8   A.   PREPARATION OF CONSTRUCTIONS

9  
10   Elaboration of Beta-Lactoglobulin Fusion Genes

11  
12   The strategy used for elaborating fusion genes  
13   comprising DNA sequence elements from the ovine  
14   beta-lactoglobulin and the gene(s) of interest to be  
15   expressed in the mammary gland is outlined in Figures 1  
16   to 10. The approach utilises sequences derived from a  
17   lambda clone, lambdaSS-1, which contains the gene for  
18   ovine beta-lactoglobulin, and whose isolation and  
19   characterisation is outlined in International Patent  
20   Application No. WO-A-8800239 (Pharmaceutical Proteins  
21   Ltd) and by Ali & Clark (1988) Journal of Molecular  
22   Biology 199, 415-426.

23  
24   The elaboration of seven constructs are described -  
25   AATB, AATA, BLG-BLG, AATC, AATD, FIXD, and DELTA-A2 in  
26   sections A1-A7 respectively. Construct AATB  
27   constitutes the primary example and the other  
28   constructs are included as comparative examples.

29  
30   The nomenclature eg AATB is generally used to describe  
31   the DNA construct without its associated bacterial  
32   (plasmid) vector sequences. This form, lacking the  
33   vector sequences, corresponds to that microinjected

1 into fertilised eggs and subsequently incorporated into  
2 the chromosome(s) of the embryo.

3

4 A1 AATB - Construction of pIII-15BLGqAAT

5

6 The construct AATB is a hybrid gene which contains  
7 sequence elements from the 5'-flanking region of the  
8 ovine beta-lactoglobulin gene fused to sequences from  
9 the human gene for alpha<sub>1</sub>-antitrypsin. The features of  
10 the AATB construct are summarised in Figure 6. The  
11 sequences from the ovine beta-lactoglobulin gene are  
12 contained in a SalI - SphI fragment of about 4.2kb  
13 which contains (by inspection) a putative 'CCAAT box'  
14 (AGCCAAGTG) [see Ali & Clark (1988) Journal of  
15 Molecular Biology 199, 415-426]. In addition there are  
16 ovine BLG sequences from this SphI to a PvuII site in  
17 the 5'-untranslated region of the BLG transcription  
18 unit. The sequence of this SphI - PvuII fragment is  
19 shown in Figure 5. This latter fragment contains a  
20 putative 'TATA box' (by inspection) [see Ali & Clark  
21 (1988) Journal of Molecular Biology 199, 415-426]. The  
22 mRNA cap site / transcription start point CACTCC as  
23 determined by S1-mapping and RNase protection assays is  
24 also contained within this fragment. Beyond the fusion  
25 (PvuII-0) site are found sequences from a cDNA for  
26 human alpha<sub>1</sub>-antitrypsin and from the human  
27 alpha<sub>1</sub>-antitrypsin gene. The sequences from the 5'  
28 fusion (TagI-0) site to the BamHI site 80 bp  
29 downstream, include the initiation ATG methionine codon  
30 for alpha<sub>1</sub>-antitrypsin. The first nucleotide  
31 (cytosine) in the AAT sequences (CGACAATG..., see  
32 Figure 5) corresponds to the last nucleotide in exon I  
33 of the AAT gene. The second nucleotide (guanosine) in

1 the AAT sequences (CGACAATG..., see Figure 5)  
2 corresponds to the first nucleotide in exon II of the  
3 AAT gene. The exclusion of intron I has been effected  
4 by using DNA from a cDNA clone p8 $\alpha$ 1ppg (see below) as  
5 the source of the first 80 bp of the AAT sequences in  
6 AATB (TagI-0 to BamHI). The BamHI site corresponds to  
7 that found in exon II of the AAT gene. Beyond this  
8 BamHI site are approximately 6.5 kb of the human AAT  
9 gene including - the rest of exon II, intron II, exon  
10 III, intron III, exon IV, intron IV, exon V and about  
11 1.5 kb of 3'-flanking sequences. Exon V contains the  
12 AAT translation termination codon (TAA) and the  
13 putative polyadenylation signal (ATTAAA). The signal  
14 peptide for the peptide encoded by construct AATB is  
15 encoded by the AAT cDNA sequence from ATGCCGTCT to  
16 TCCCTGGCT (2 bp upstream from the BamHI site in exon  
17 II.

18

19 Plasmid pSS1tgSE $\alpha$ 1AT

20 The subclone pSS1tgSE $\alpha$ 1AT was constructed as described  
21 here and briefly in Example 2 of International Patent  
22 Application No. WO-A-8800239 (Pharmaceutical Proteins  
23 Ltd). This clone contains the cDNA sequences for human  
24  $\alpha_1$ -antitrypsin inserted into the 5'-untranslated  
25 region of the ovine beta-lactoglobulin gene. The  
26 plasmid p8 $\alpha$ 1ppg containing a full length cDNA encoding  
27 an M variant of  $\alpha_1$ -antitrypsin was procured from  
28 Professor Riccardo Cortese, European Molecular Biology  
29 Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg,  
30 Federal Republic of Germany (Ciliberto, Dente & Cortese  
31 (1985) Cell 41, 531-540). The strategy used in the  
32 construct BLG-AAT or pSS1tgXSTARG, now known as AATA,  
33 described in International Patent Application No.

1 WO-A-8800239 (Pharmaceutical Proteins Ltd) required  
2 that the polyadenylation signal sequence at the 3' end  
3 of the  $\alpha_1$ -antitrypsin cDNA be removed.

4  
5 The polyadenylation signal was removed in the following  
6 manner. Plasmid p8 $\alpha$ lppg DNA was digested with PstI and  
7 the digestion products were separated by  
8 electrophoresis in a preparative 1% agarose gel  
9 containing 0.5  $\mu$ g/ml ethidium bromide (Sigma). The  
10 relevant fragment of about 1400 bp was located by  
11 illumination with a UV lamp (Ultra-Violet Products,  
12 Inc, San Gabriel, California, USA). A piece of  
13 dialysis membrane was inserted in front of the band and  
14 the DNA fragment subsequently electrophoresed onto the  
15 membrane. The DNA was eluted from the dialysis  
16 membrane and isolated by use of an 'ElutipD' [Scleicher  
17 and Schull, Postfach 4, D-3354, Dassel, W. Germany],  
18 employing the procedure recommended by the  
19 manufacturer. The gel purified 1400 bp PstI fragment  
20 was digested with the TaqI, electrophoresed on a  
21 preparative 1% agarose gel as described above. The  
22 TaqI - PstI fragment of approximately 300 bp comprising  
23 the 3' end of the  $\alpha_1$ -antitrypsin cDNA including the  
24 polyadenylation signal sequence was eluted and purified  
25 using an Elutip as described above, as was the TaqI -  
26 TaqI fragment of 1093 bp containing the 5' portion of  
27 the cDNA. The plasmid vector pUC8 (Pharmacia-LKB  
28 Biotechnology, Pharmacia House, Midsummer Boulevard,  
29 Central Milton Keynes, Bucks, MK9 3HP, UK) was digested  
30 with AccI and PstI, phenol/chloroform extracted and DNA  
31 recovered by ethanol precipitation. The 300 bp TaqI -  
32 PstI fragment from p8 $\alpha$ lppg was ligated using T4 DNA  
33 ligase to pUC8 cut with AccI and PstI and the ligation

1 products were used to transform E. coli strain DH-1  
2 (Gibco-BRL, PO Box 35, Trident House, Renfrew Road,  
3 Paisley PA3 4EF, Scotland, UK) to ampicillin  
4 resistance. Plasmid DNA was isolated from ampicillin  
5 resistant colonies. The correct recombinants were  
6 identified by the release of a fragment of  
7 approximately 300 bp on double digestion with AccI and  
8 PstI. The plasmid generated was called pUC8.3'AT.3.

9  
10 Plasmid pUC8.3'AT.3 was subjected to partial digestion  
11 with BstNI and the fragment(s) corresponding to  
12 linearised pUC8.3'AT.3 isolated from an agarose gel.  
13 There are seven BstNI sites in pUC.3'AT.3, five in the  
14 vector and two in the region corresponding to the  
15 3'-untranslated sequences of alpha<sub>1</sub>-antitrypsin. The  
16 BstNI linearised and gel purified DNA was digested with  
17 PstI which cuts in the pUC8 polylinker where it joins  
18 the 3' end of the cDNA insert. The PstI digested DNA  
19 was end repaired with T4 DNA polymerase in the presence  
20 of excess dNTPs and self-ligated with T4 DNA ligase.  
21 The BstNI - PstI fragment containing the  
22 polyadenylation signal sequence is lost by this  
23 procedure. The ligated material was used to transform  
24 E. coli strain DH-1 to ampicillin resistance. Plasmid  
25 DNA was isolated from ampicillin resistant colonies.  
26 The correct clone was identified by restriction  
27 analysis and comparison with pUC8.3'AT.3. The correct  
28 clone was characterised by retention of single sites  
29 for BamHI and HindIII, loss of a PstI site, and a  
30 reduction in the size of the small PvuII fragment. The  
31 correct clone was termed pB5.

32

33



1 Plasmid pB5 DNA was digested with AccI,  
2 phenol/chloroform extracted and DNA recovered by  
3 ethanol precipitation. AccI cleaved pB5 DNA was  
4 treated with calf intestine alkaline phosphatase (BCL).  
5 The reaction was stopped by adding EDTA to 10  
6 millimolar and heating at 65°C for 10 minutes. The DNA  
7 was recovered after two phenol/chloroform and one  
8 chloroform extractions by precipitation with ethanol.  
9 T4 DNA ligase was used to ligate the 1093 bp TaqI -  
10 TaqI fragment described above to pB5, AccI cleaved and  
11 phosphatased DNA and the ligation products were used to  
12 transform E. coli strain HB101 (Gibco-BRL) to  
13 ampicillin resistance. The identity of the correct  
14 clone (pUC8 $\alpha$ 1AT.73) was verified by restriction  
15 analysis - presence of a 909 bp HinfI fragment, a 1093  
16 bp TaqI fragment, and a 87 bp BamHI fragment.

17

18 The  $\alpha_1$ -antitrypsin cDNA minus its polyadenylation  
19 signal was excised from pUC8 $\alpha$ 1AT.73 as a 1300 bp AccI -  
20 HindIII fragment and isolated from a preparative gel.  
21 The 1300 bp AccI - HindIII fragment was end-repaired  
22 with the Klenow fragment of E. coli DNA polymerase in  
23 the presence of excess dNTPs. The fragment was ligated  
24 into PvuII restricted, phosphatase treated pSS1tgSE DNA  
25 (see International Patent Application No. WO-A-8800239  
26 (Pharmaceutical Proteins Ltd) to form pSS1tgSE $\alpha$ 1AT  
27 after transforming E. coli DH-1 to ampicillin  
28 resistance.

29

30 Plasmid pIII-ISpB (see Figure 1)  
31 pSS1tgSE $\alpha$ 1AT DNA was linearised by digestion with SphI  
32 which cuts at a unique site in the plasmid in a region  
33 of DNA corresponding to the 5' flanking sequences of

1 the beta-lactoglobulin transcription unit. The DNA was  
2 recovered after phenol/chloroform extractions by  
3 precipitation with ethanol. The SphI linearised  
4 plasmid was digested with BamHI which cuts at a unique  
5 site in the plasmid in a region of DNA corresponding to  
6 the mRNA sequences of alpha<sub>1</sub>-antitrypsin. The 155 bp  
7 SphI - BamHI fragment, comprising beta-lactoglobulin  
8 sequences fused to alpha<sub>1</sub>-antitrypsin sequences was  
9 located in a 1% agarose gel and isolated by use of an  
10 ElutipD as described above.

11

12 The plasmid pIII-ISpB was constructed by using T4 DNA  
13 ligase to ligate the 155 bp SphI - BamHI fragment from  
14 subclone pSS1tgSEα1AT into the plasmid vector  
15 pPolyIII-I (Lathe, Vilotte & Clark, 1987, Gene 57,  
16 193-201) which had been digested with SphI and BamHI.  
17 [The vector pPolyIII-I is freely available from  
18 Dr. A. J. Clark, AFRC Institute of Animal Physiology  
19 and Genetics Research, West Mains Road, Edinburgh EH9  
20 3JQ, UK.] Clones were isolated after transforming  
21 competent E. coli DH5α cells (Gibco-BRL) to ampicillin  
22 resistance. Plasmid DNA was prepared from the  
23 ampicillin resistant colonies and screened for the  
24 desired product. pIII-ISpB was confirmed as the  
25 desired product by the retention of cleavage sites for  
26 the enzymes BamHI and SphI and by the addition (when  
27 compared to the vector pPolyIII-I) of a cleavage site  
28 for the enzyme StuI. The StuI site is present in the  
29 155 bp SphI - BamHI fragment isolated from  
30 pSS-1tgSEα1AT.

31

32 Plasmid pIII-15BLGSpB (pAT2-3) (see Figure 2)  
33 pIII-ISpB DNA was digested with the SphI and SalI.

1    SphI cuts at a unique site in the plasmid in a region  
2    of DNA corresponding to the 5' flanking sequences of  
3    the beta-lactoglobulin transcription unit. This site  
4    represents the junction between the beta-lactoglobulin  
5    sequences and the plasmid vector sequences. SalI cuts  
6    at a unique site in the plasmid in the vector  
7    polylinker sequences. The SphI/SalI digested pIII-ISpB  
8    DNA was electrophoresed on a preparative 1% agarose gel  
9    as described above. The SalI - SphI fragment of  
10   approximately 2.2 kb was eluted and purified using an  
11   Elutip as described above.

12

13   The plasmid DNA pSS-1tgXS (described in International  
14   Patent Application No. WO-A-8800239 (Pharmaceutical  
15   Proteins Ltd)) was digested with SphI and SalI and the  
16   DNA electrophoresed on a 0.9% agarose gel. The  
17   relevant SalI - SphI fragment, comprising approximately  
18   4.2 kb of DNA sequences from the 5' flanking sequences  
19   of the beta-lactoglobulin gene, was located by  
20   illumination with ultra violet light and recovered by  
21   use of an Elutip as described above.

22

23   The plasmid pIII-15BLGSpB was constructed by using T4  
24   DNA ligase to ligate the 4.2 kb SalI - SphI fragment  
25   described above into gel purified SalI - SphI digested  
26   pIII-ISpB DNA. Clones were isolated after transforming  
27   E. coli DH5 $\alpha$  (Gibco-BRL) to ampicillin resistance.  
28   Plasmid DNA was prepared from the ampicillin resistant  
29   colonies and screened for the desired product. The  
30   correct product was verified by the presence of two  
31   BamHI sites - one in the 4.2 kb fragment containing the  
32   5' flanking sequences of beta-lactoglobulin and one in  
33   the sequences corresponding to the  $\alpha_1$ -antitrypsin

1 mRNA. Cleavage of the correct product with BamHI  
2 yields two fragments including one of approximately  
3 1.75 kb which spans the cloning junctions (see  
4 Figure 2).

5

6 Plasmid pIII-15BLGgAAT (AATB or G7) (see Figure 3)

7 An alpha<sub>1</sub>-antitrypsin DNA clone pATp7 was procured from  
8 Dr. Gavin Kelsey, MRC Human Biochemical Genetics Unit,  
9 The Galton Laboratory, University College London,  
10 Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.  
11 This clone contains the entire alpha<sub>1</sub>-antitrypsin  
12 transcription unit plus 348 bp of 5' and approximately  
13 1500 bp of 3' flanking sequences as an insert of  
14 approximately 12.3 kb in the BamHI site of a plasmid  
15 vector pUC9 (Pharmacia-LKB Biotechnology, Pharmacia  
16 House, Midsummer Boulevard, Central Milton Keynes,  
17 Bucks, MK9 3HP, UK). The insert for clone pATp7 was  
18 prepared by partial BamHI and partial BglIII digestion  
19 of cosmid clone  $\alpha$ ATc1 (Kelsey, Povey, Bygrave &  
20 Lovell-Badge (1987) Genes and Development 1, 161-171).  
21 The clone pATp7 contains the gene which encodes the M<sub>1</sub>  
22 allele, which is the most frequent at the Pi locus.  
23 Most of the DNA sequence of this gene is reported by  
24 Long, Chandra, Woo, Davie & Kurachi (1984) Biochemistry  
25 23, 4828-4837.

26

27 Plasmid DNA from pATp7 was digested with BamHI and  
28 electrophoresed in a 0.9% agarose gel. The relevant  
29 BamHI fragment, comprising approximately 6500bp of  
30 alpha<sub>1</sub>-antitrypsin sequences from the BamHI site in  
31 exon II of this gene to a BamHI site in the 3' flanking  
32 region was located and purified by use of an Elutip as  
33 described above.

1 The plasmid pIII-15BLGSpB (also known as AT2-3) was  
2 linearised by partial digestion with BamHI. There are  
3 two BamHI sites in this plasmid one in the sequences  
4 corresponding to the 5' flanking sequences of  
5 beta-lactoglobulin and the other in the sequences  
6 corresponding to the mRNA for alpha<sub>1</sub>-antitrypsin. The  
7 latter site is the desired site for insertion of the  
8 6500 bp BamHI fragment from pATp7. The products of the  
9 partial BamHI digestion of plasmid pIII-15BLGSpB were  
10 electrophoresed in a 0.9% agarose gel. The fragment(s)  
11 corresponding to linearised pIII-15BLGSpB were located  
12 and purified using an Elutip as described above. It is  
13 expected that this fragment preparation will contain  
14 the two possible BamHI linearised molecules. BamHI  
15 linearised, gel purified DNA was dissolved in TE (10 mM  
16 Tris.HCl, 1 mM EDTA pH 8) and treated with calf  
17 intestinal phosphatase (BCL) for 30 minutes at 37°C.  
18 The reaction was stopped by adding EDTA to 10  
19 millimolar and heating at 65°C for 10 minutes. The DNA  
20 was recovered after two phenol/chloroform and one  
21 chloroform extractions by precipitation with ethanol.

22  
23 The plasmid pIII-15BLGgAAT was constructed by using T4  
24 DNA ligase to ligate the 6500 bp BamHI fragment from  
25 pATp7 into BamHI linearised, gel purified and  
26 phosphatase treated pIII-15BLGSpB DNA. Clones were  
27 isolated after transforming E. coli DH-5 (Gibco-BRL) to  
28 ampicillin resistance. Plasmid DNA was purified from  
29 the ampicillin resistant colonies and screened for the  
30 desired product. The desired clones were characterised  
31 by restriction analysis and, in particular, by the  
32 presence of an SphI fragment of approximately 1.6 kb.  
33 Plasmid DNA was prepared for one such clone (G7) and

1 given the nomenclature pIII-15BLGgAAT (also known as  
2 AATB).

3  
4 The diagnostic 1.6kb SphI fragment was subcloned from  
5 pIII-15BLGgAAT into the SphI site of the M13 vector  
6 M13tg130 (Kieny, Lathe & Lecocq (1983) Gene 26, 91-99).  
7 The DNA sequence of 180 nucleotides from the SphI site  
8 corresponding to that in the 5' flanking region of the  
9 beta-lactoglobulin gene in a 3' direction through the  
10 fusion point of the beta-lactoglobulin and  
11 alpha<sub>1</sub>-antitrypsin sequences was determined by the  
12 chain terminator reaction using a Sequenase<sup>TM</sup> kit (USB,  
13 United States Biochemical Corporation, PO Box 22400,  
14 Cleveland, Ohio 44122, USA) according to the  
15 manufacturers instructions. The sequence of this  
16 region is given in Figure 5.

17

18 Preparation of DNA for microinjection (see Figure 4)  
19 The  $\beta$ -lactoglobulin/ $\alpha$ 1-antitrypsin fusion gene insert  
20 was excised from pIII-15BLGgAAT as follows. 25-50  $\mu$ g  
21 aliquots of pIII-15BLGgAAT plasmid DNA were digested  
22 with NotI and the digested material electrophoresed on  
23 a 0.6% agarose gel. The larger fragment of  
24 approximately 10.5 kb was visualised under ultra-violet  
25 light and purified using an Elutip as described above.  
26 Following ethanol precipitation of the DNA eluted from  
27 the Elutip, the DNA was further purified as follows.  
28 The DNA was extracted once with phenol/chloroform, once  
29 with chloroform and was then precipitated with ethanol  
30 twice. The DNA was washed with 70% ethanol, dried  
31 under vacuum and dissolved in TE (10 mM Tris.HCl, 1mM  
32 EDTA pH 8). All aqueous solutions used in these later  
33 stages had been filtered through a 0.22  $\mu$ m filter.

1 Pipette tips were rinsed in filtered sterilised water  
2 prior to use. The DNA concentration of the purified  
3 insert was estimated by comparing aliquots with known  
4 amounts of bacteriophage lambda DNA on ethidium bromide  
5 stained agarose gels. The insert DNA was checked for  
6 purity by restriction mapping.

7

8 **A2 AATA - Construction of pSS1tgXS $\alpha$ 1AT**

9

10 The construct AATA is analogous to the construct  
11 BLG-FIX or pSS1tgXSFIX described in International  
12 Patent Application No. WO-A-8800239 (Pharmaceutical  
13 Proteins Ltd). The elaboration of AATA is outlined in  
14 Example 2 of International Patent Application No.  
15 WO-A-8800239 (Pharmaceutical Proteins Ltd) as a second  
16 example of the generalised construct pSS1tgXSTARG. The  
17 first stages of the construction of AATA (ie the  
18 generation of the plasmid pSS1tgSE $\alpha$ 1AT) are described  
19 above in section A1,

20

21 **A3 BLG-BLG - Construction of pSS1tgXSDELTA $\alpha$ 1aBLG** (see  
22 Figures 7 and 8)

23

24 The construct is analogous to FIXA and AATA (generally  
25 designated as pSS1tgXSTARG and specifically as BLG-FIX  
26 and BLG-AAT in patent WO-A-8800239) ie, the cDNA for  
27 ovine  $\beta$ -lactoglobulin has been inserted into the PvuII  
28 site in the first exon of pSS1tgXSDELTA $\alpha$ 1a (see below).  
29 pSS1tgXSDELTA $\alpha$ 1a is a variant of pSS1tgXS lacking the  
30 ClaI restriction site found in exon 3 which should  
31 cause a frameshift in the 2nd open reading frame in the  
32 expected bicistronic message of BLG-BLG and premature  
33 termination of any polypeptide being translated. It

1 was necessary to sabotage the 2nd open reading frame in  
2 this manner in order that the polypeptides encoded by  
3 the two open reading frames could be distinguished. In  
4 order to generate this construct a full length BLG cDNA  
5 had first to be made.

6

7 pUCBlacA

8 Two complimentary 44-mer oligonucleotides, synthesised  
9 by the Oswell DNA Service, Department of Chemistry,  
10 University of Edinburgh, and containing bases 117-159  
11 of the ovine  $\beta$ -lactoglobulin cDNA sequence (Gaye *et al*,  
12 (1986) *Biochimie* 68, 1097-1107) were annealed to  
13 generate SalI and StyI complimentary termini. The  
14 annealed oligonucleotides were then ligated using T4  
15 DNA ligase to equimolar amounts of a gel purified 457  
16 bp StyI - SmaI fragment from  $\beta$ -Lg 931 (Gaye *et al*, *op*  
17 *cit*) and gel purified pUC19 (Pharmacia-LKB  
18 Biotechnology, Pharmacia House, Midsummer Boulevard,  
19 Central Milton Keynes, Bucks, MK9 3HP, UK) which had  
20 been digested with SalI - SmaI. After transformation  
21 of competent *E. coli* strain JM83 (see Messing (1979)  
22 Recombinant DNA Technical Bulletin, NIH Publication No.  
23 79-99, 2, No. 2 (1979), 43-48) the correct recombinant  
24 was determined by restriction analysis.

25

26 pUCBlacB

27 pUCBlacA digested with SphI and StuI was ligated to  
28 equimolar amounts of a gel purified 163 bp SphI - StuI  
29 fragment from pSSltgSE (described in patent  
30 WO-A-8800239) using T4 DNA ligase. After  
31 transformation of competent *E. coli* strain JM83 the  
32 correct recombinant was determined by restriction  
33 analysis.



## 1 pSS1tgXSDELTAcl

2 After transformation of competent E. coli strain DL43  
3 (relevant phenotype  $\text{dam}^-$ ,  $\text{dcm}^-$ ; also called GM119, gift  
4 of Dr. D. Leach, Department of Molecular Biology,  
5 University of Edinburgh, West Mains Road, Edinburgh  
6 EH9, UK) with the plasmid pSS1tgXS plasmid DNA was  
7 isolated and digested to completion with ClaI. The DNA  
8 termini were end-repaired using the Klenow fragment of  
9 E. coli DNA polymerase in the presence of excess dNTP's  
10 prior to ligation with T4 DNA ligase in the presence of  
11 1mM hexamine cobalt chloride, 25mM KCl ([to encourage  
12 self-ligation (Rusche & Howard-Flanders (1985) Nucleic  
13 Acids Research 13, 1997-2008)]). The ligation products  
14 were used to transform competent DL43 and ClaI  
15 deficient recombinants were confirmed by restriction  
16 analysis.

17

## 18 pSS1tgSE\_BLG

19 Equimolar amounts of gel purified pSS1tgSE, digested to  
20 completion with PvuII and dephosphorylated with Calf  
21 intestinal phosphatase (BCL), were ligated to a gel  
22 purified 580 bp PvuII - SmaI fragment from pUC $\Delta$ lacB  
23 using T4 DNA ligase. After transformation of competent  
24 DH5 $\alpha$  (Gibco-BRL) the correct recombinant was confirmed  
25 by restriction analysis.

26

## 27 pSE\_BLG\_3'

28 Equimolar amounts of gel purified pSS1tgSE\_BLG digested  
29 to completion with EcoRI were ligated to 3 (~4.3-5.3)  
30 gel purified products of a partial EcoRI digestion of  
31 pSS1tgXSDELTAcl using T4 DNA ligase. After  
32 transformation of competent DH5 $\alpha$  (Gibco-BRL) the  
33 correct recombinant was identified by restriction  
34 analysis.

1 pSSltgXSDELTAclabLG

2 The gel purified ~3 kb SphI - HindIII fragment from  
3 pSE\_BLG\_3' was ligated to equimolar amounts of gel  
4 purified ~9.6 kb SphI-HindIII fragment from  
5 pSSltgDELTASphXS (a derivative of pSSltgXS lacking the  
6 SphI restriction site in the polylinker region of the  
7 vector pPoly1) using T4 DNA ligase. After  
8 transformation of competent DL43 the construct was  
9 confirmed by restriction analysis.

10

11 Isolation of DNA fragment for microinjection

12 pSSltgXSDELTAclabLG was digested to completion with  
13 BqIII and XbaI to pSSltgXSDELTAclabLG was digested to  
14 completion with BqIII and XbaI to liberate the insert  
15 from the vector. The insert was recovered from an  
16 agarose gel by electroelution onto dialysis membrane  
17 (Smith (1980) Methods in Enzymology 65, 371-380).  
18 After release from the membrane the DNA was  
19 phenol/chloroform extracted, ethanol precipitated and  
20 resuspended in 100  $\mu$ l H<sub>2</sub>O ready for microinjection.

21

22 A4 AATC - Construction of pSSlpUCXSTGA.AAT (see  
23 Figure 9)

24

25 This construct contains the cDNA sequences encoding  
26 human alpha-1-antitrypsin (AAT) inserted into the  
27 second exon of the ovine  $\beta$ -lactoglobulin (BLG) gene.  
28 The aim was to determine whether or not inserting the  
29 AAT cDNA sequences at a site distant from the BLG  
30 promoter would improve the levels of expression. As  
31 such, this construct comprises the intact first exon  
32 and first intron of the BLG gene.

33

1 Since this construct contains two ATG codons (including  
2 the normal BLG initiating methionine) in the first BLG  
3 exon (ie before the sequences encoding AAT) an  
4 'in-frame' termination codon (TGA) was introduced at  
5 the junction point between BLG and AAT. This was  
6 thought necessary to prevent the production of a fusion  
7 protein between BLG and AAT. It will be noted that for  
8 AAT protein to be produced from the expected  
9 transcripts, reinitiation(at the natural initiating ATG  
10 of AAT) of transcription will have to take place after  
11 termination at this codon.

12

13 pSSltgSE.TGA

14 Two oligonucleotides (5'CTTGTGATATCG3' and  
15 5'AATTCGATATCAC3') were synthesised by the Oswell DNA  
16 Service, Department of Chemistry, University of  
17 Edinburgh. After annealing, the oligonucleotides  
18 comprise a TGA stop codon, an EcoRV site and have  
19 cohesive ends for a StyI and an EcoRI site,  
20 respectively. The annealed oligonucleotides were  
21 ligated to a gel purified StyI-EcoRI fragment of about  
22 3.2 kb isolated from pSSltgSE (pSSltgSE is described in  
23 International Patent Application No. WO-A-8800239  
24 (Pharmaceutical Proteins ltd)). This will insert these  
25 sequences at the StyI site which comprises nucleotides  
26 20-25 of BLG-exon II and generates the plasmid  
27 pSSltgSE.TGA, in which the TGA stop codon is 'in frame'  
28 with the sequences encoding BLG. Note the sequences 3'  
29 to the BLG StyI site are replaced by the  
30 oligonucleotides in this step. The ligation products  
31 were used to transform E.coli strain DH5 $\alpha$  (Gibco-BRL)  
32 to ampicillin resistance. The correct clone  
33 (pSSltgSE.TGA) was identified by restriction analysis -

1 retention of sites for EcoRI and SphI and acquisition  
2 of a site for EcoRV.

3

4 pSSltgSpX.TGA

5 pSSltgSE.TGA was cleaved with EcoRI and the cohesive  
6 termini were end-repaired by filling in with Klenow  
7 fragment of E. coli DNA polymerase in the presence of  
8 excess dNTPs. After end-repair the preparation was  
9 cleaved with SphI and the insert fragment of about  
10 800 bp (now SphI->EcoRI (blunt)) was isolated on a  
11 preparative gel. Plasmid pBJ7 (this patent, see below,  
12 section A4) was cleaved with SphI and PvuII and the  
13 larger (about 4.3 kb) fragment isolated. Note that  
14 this fragment contains the pPolyI vector sequences.  
15 The SphI-EcoRI (blunt) fragment excised from  
16 pSSltgSE.TGA was ligated using T4 DNA ligase to the  
17 SphI-PvuII fragment isolated from pBJ7 and the ligation  
18 products used to transform E. coli strain DH5 $\alpha$   
19 (Gibco-BRL) to ampicillin resistance. The correct  
20 recombinant plasmid pSSltgSpX.TGA, which contains exon  
21 I, intron I, part exon II, oligonucleotide, part exon 5  
22 and exons 6 and 7 of the BLG gene, was identified by  
23 restriction analysis.

24

25 pSSlpUCXS.TGA

26 The BLG 5' SaII - SphI fragment of about 4.2 kb was  
27 isolated from pSSltgXS (WO-A-8800239) and ligated to  
28 equimolar amounts of the SphI-XbaI insert from  
29 pSSltgSpX.TGA and SaII-XbaI cleaved plasmid vector  
30 pUC18 (Pharmacia-LKB Biotechnology, Pharmacia House,  
31 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9  
32 3HP, UK). The ligation products were used to transform  
33 E. coli strain DH5 $\alpha$  (Gibco-BRL) to ampicillin

1 resistance. The correct clone, pSS1pUCXS.TGA, was  
2 identified by restriction analysis.

3  
4 pSS1pUCXSAAT.TGA (AATC)  
5 pSS1pUCXS.TGA contains a unique EcoRV site (derived  
6 from the oligonucleotide) inserted in the second exon  
7 which will cleave this plasmid 1 bp downstream of the  
8 'in-frame' TGA. cDNA sequences can thus be inserted  
9 into this plasmid downstream of the BLG sequences in  
10 the second exon. This is exemplified by the  
11 construction of pSS1pUCXSAAT.TGA (AATC) in which AccI -  
12 HindIII fragment derived from pUC8 $\alpha$ 1AT.73 (this patent,  
13 see Section A1 above) was inserted at the EcoRV site.  
14 Plasmid pUC8 $\alpha$ 1AT.73 (described in section A1 above) was  
15 digested with AccI and HindIII and the resulting  
16 fragment containing the alpha<sub>1</sub>-antitrypsin cDNA minus  
17 its polyadenylation signal was end-repaired using  
18 Klenow fragment of E. coli DNA polymerase in the  
19 presence of excess dNTPs. This blunt ended fragment  
20 was gel purified and ligated using T4 DNA ligase to gel  
21 purified pSS1pUCXS.TGA cleaved with EcoRV and  
22 dephosphorylated to prevent recircularisation. After  
23 transformation of competent E. coli strain DH5 $\alpha$   
24 (Gibco-BRL) with the ligation products, the correct  
25 clone was identified by restriction enzyme analysis.

26

27 A5 Construction of AATD (pBJ16) (see Figure 10)

28 This construct contains the cDNA for human  
29 alpha<sub>1</sub>-antitrypsin flanked by BLG sequences. The 5'  
30 flanking sequences include the SalI to PvuII-0 BLG  
31 sequences also present in AATA and AATB. The fusion  
32 point between the BLG and AAT sequences is in the  
33 5'-untranslated region of the BLG first exon as is the

1 case in AATA, FIXA and AATB. The 3' flanking sequences  
2 comprise exons 6 and 7 of BLG and the 3' flanking  
3 sequences of the BLG gene as far as the XbaI site.  
4 This construct contains no introns and was designed to  
5 examine whether the 5' and 3' BLG sequences described  
6 above are sufficient to direct efficient mammary  
7 specific expression of cDNAs encoding human plasma  
8 proteins as exemplified by that for AAT.

9

10 Plasmid pSSltgSpX

11 The gel purified SphI - XbaI restriction fragment of  
12 about 6.6 kb from pSSltgXS (described in patent  
13 WO-A-8800239) was ligated using T4 DNA ligase to gel  
14 purified pPolyI (Lathe, Vilotte & Clark, 1987, Gene 57,  
15 193-201) (also described in patent WO-A-8800239)  
16 digested with SphI and XbaI. [The vector pPolyI is  
17 freely available from Professor R. Lathe, LGME-CNRS and  
18 U184 INSERM, 11 rue Humann, 67085, Strasbourg, France.]  
19 After transformation of competent E. coli strain DHR $\alpha$   
20 (Gibco-BRL) the correct clone was identified by  
21 restriction enzyme analysis.

22

23 Plasmid pBJ5

24 The gel purified PvuII restriction fragment containing  
25 the origin of replication from pSSltgSpX was  
26 self-ligated using T4 DNA ligase in the presence of 1mM  
27 hexamine cobalt chloride, 25mM KCl [to encourage  
28 self-ligation (Rusche & Howard-Flanders (1985) Nucleic  
29 Acids Research 13, 1997-2008)]. After transformation  
30 of competent E. coli strain DHR $\alpha$  (Gibco-BRL) the  
31 correct clone was identified by restriction enzyme  
32 analysis.

33

1 Plasmid pUCBlacA

2 See example 1 A3 for a description of pUCBlacA

3

4 Plasmid pBJ7

5 The gel purified HincII - SmaI restriction fragment  
6 from pUCBlacA was ligated using T4 DNA ligase to gel  
7 purified pBJ5 linearised by partial digestion with  
8 SmaI. After transformation of competent E. coli strain  
9 DH5 $\alpha$  (Gibco-BRL) the correct clone was identified by  
10 restriction enzyme analysis.

11

12 Plasmid pBJ8

13 The gel purified PvuII restriction fragment containing  
14 the origin of replication from pBJ7 was self-ligated  
15 using T4 DNA ligase in the presence of 1mM hexamine  
16 cobalt chloride, 25mM KCl (to encourage self-ligation  
17 [Rusche & Howard-Flanders (1985) Nucleic Acids Research  
18 13, 1997-2008]). After transformation into competent  
19 E. coli strain DH5 $\alpha$  (Gibco-BRL) the correct clone was  
20 identified by restriction enzyme analysis.

21

22 Plasmid pBJ12

23 Plasmid pUC8 $\alpha$ 1AT.73 (described in section A1 above) was  
24 digested with AccI and HindIII and the resulting  
25 fragment containing the  $\alpha_1$ -antitrypsin cDNA minus  
26 its polyadenylation signal was end-repaired using  
27 Klenow fragment of E. coli DNA polymerase in the  
28 presence of excess dNTPs. This blunt ended fragment  
29 was gel purified and ligated using T4 DNA ligase to gel  
30 purified pBJ8 linearised with PvuII. After  
31 transformation of competent E. coli strain DH5 $\alpha$   
32 (Gibco-BRL) the correct clone was identified by  
33 restriction enzyme analysis.

1 Plasmid pBJ1  
2 Plasmid pSS1tgSpS (described in this patent, see A7  
3 below) was digested with BqIII and end-repaired using  
4 the Klenow fragment of E. coli DNA polymerase in the  
5 presence of excess dNTPs. The blunt-ends were modified  
6 using HindIII synthetic linkers (New England Biolabs  
7 Inc, 32 Tozer Road, Beverly, MA 01915-5510, USA) and  
8 the resulting fragment self-ligated using T4 DNA ligase  
9 in the presence of 1mM hexamine cobalt chloride, 25mM  
10 KCI (to encourage self-ligation [Rusche &  
11 Howard-Flanders (1985) Nucleic Acids Research 13,  
12 1997-2008]). After transformation of competent E. coli  
13 strain DH5 $\alpha$  (Gibco-BRL) the correct clone was  
14 identified by restriction enzyme analysis.

15  
16 Plasmid pBJ16 (AATD)  
17 The gel purified HindIII - SphI fragment from pBJ1 and  
18 the gel purified SphI - XbaI fragment from pBJ12 were  
19 ligated using T4 DNA ligase to gel purified pUC19  
20 (Pharmacia-LKB Biotechnology, Pharmacia House,  
21 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9  
22 3HP, UK) digested with HindIII and XbaI. After  
23 transformation of competent E. coli strain DH5 $\alpha$   
24 (Gibco-BRL) the correct clone was identified by  
25 restriction enzyme analysis.

26  
27 Isolation of AAT-D fragment from pBJ16 for  
28 microinjection

29 Plasmid pBJ16 was digested with HindIII and XbaI and  
30 the resulting 8.0 kb AATD fragment was isolated from a  
31 gel using DE81 paper (Dretzen et al (1981) Analytical  
32 Biochemistry 112, 285-298). After separation from the  
33 DE81 paper the DNA was phenol/chloroform extracted,



1 ethanol precipitated and finally resuspended in TE  
2 buffer (10 mM Tris-HCl, 1mM EDTA pH 8) ready for  
3 microinjection.

4

5 **A6** FIXD - Construction of pBJ17

6

7 The procedure of Example 1 A5 (construction of AATD) is  
8 repeated, except that the DNA sequence encoding the  
9 polypeptide of interest encodes Factor IX. A NheI -  
10 HindIII fragment comprising 1553 bp of the insert from  
11 p5'G3'CVI [see International Patent Application No.  
12 WO-A-8800239 (Pharmaceutical Proteins Ltd)] was  
13 inserted into the PvuII site of pBJ8 as described above  
14 for pBJ12.

15

16 **A7** DELTA-A2 - Construction of pSSltgXDELTA-AvaII  
17 (DELTA A2)

18

19 This construct contains the minimum ovine  
20 beta-lactoglobulin sequences that have so far been  
21 shown in transgenic mice to result in tissue-specific  
22 expression of the protein during lactation. The  
23 complete sequence of this construct can be found in  
24 Harris, Ali, Anderson, Archibald & Clark (1988),  
25 Nucleic Acids Research 16 (in press).

26

27 Plasmid pSSltgSpS

28 The gel purified SalI - SphI restriction fragment of  
29 approximately 4.2 kb isolated from pSSltgXS (described  
30 in patent WO-A-8800239) was ligated, using T4 DNA  
31 ligase, with equimolar amounts of gel purified pPolyI  
32 (Lathe, Vilotte & Clark, 1987, Gene 57, 193-201)  
33 digested with SalI and SphI. [The vector pPolyI is

1 freely available from Professor R. Lathe, LGME-CNRS and  
2 U184 INSERM, 11 rue Humann, 67085 Strasbourg, France.]  
3 After transformation of competent E. coli strain DH1  
4 (Gibco-BRL) the correct clone was identified by  
5 restriction analysis.

6  
7 Plasmid pSS1tgSpDELTA-AvaII  
8 Plasmid pSS1tgSpS was partially digested with AvaI  
9 followed by digestion to completion with SalI. The  
10 ends of the resultant DNA fragments were end-repaired  
11 using the Klenow fragment of E. coli DNA polymerase in  
12 the presence of excess dNTPs. After ligation using T4  
13 DNA ligase in the presence of 1mM hexamine cobalt  
14 chloride, 25mM KCl [to encourage self-ligation (Rusche  
15 & Howard-Flanders (1985) Nucleic Acids Research 13,  
16 1997-2008)], the DNA was used to transform competent  
17 DH1 (Gibco-BRL). The correct AvaI deletion recombinant  
18 was confirmed by restriction analysis.

19  
20 Plasmid pSS1tgXDELTA-AvaII  
21 The gel purified ~800 bp SphI - BqIII fragment from  
22 pSS1tgSpDELTA-AvaII; ~6.5 kb SphI - XbaI fragment from  
23 pSS1tgXS; and pPolyI digested with BqIII - XbaI were  
24 ligated in approximately equimolar ratios using T4 DNA  
25 ligase then used to transform competent DH1  
26 (Gibco-BRL). The identity of the correct recombinant  
27 was confirmed by restriction analysis.

28  
29 Isolation of DNA fragment for injection  
30 pSS1tgXDELTA-AvaII was digested to completion with  
31 BqIII and XbaI to release the ~7.4 kb insert from the  
32 vector. The insert was recovered from an agarose gel  
33 using DE81 paper (Dretzen et al (1981) Analytical

1 Biochemistry 112, 295-298). After separation from the  
2 DE81 paper the DNA was phenol/chloroform extracted,  
3 ethanol precipitated and resuspended in 100  $\mu$ l TE ready  
4 for microinjection. Alternatively, the insert was  
5 recovered from an agarose gel by electroelution onto  
6 dialysis membrane (Smith (1980) Methods in Enzymology  
7 65, 371-380). After release from the membrane the DNA  
8 was phenol/chloroform extracted, ethanol precipitated  
9 and resuspended in 100  $\mu$ l H<sub>2</sub>O ready for microinjection.

10

11 **B. CONSTRUCTION OF TRANSGENIC ANIMALS**

12

13 **MICE**

14

15 Procedures are similar to those described by Hogan,  
16 Costantini and Lacy in "Manipulating the Mouse Embryo:  
17 A Laboratory Manual" Cold Spring Harbor Laboratory  
18 (1986).

19

20 **Collection of fertilised eggs**

21

22 Mice used for the collection of fertilised eggs are F<sub>1</sub>  
23 hybrids between the C57BL/6 and CBA inbred strains of  
24 mice. C57BL/6 females and CBA males are obtained from  
25 Harlan Olac Ltd (Shaw's Farm, Bicester OX6 OTP,  
26 England) and used for the breeding of F<sub>1</sub> hybrids. The  
27 mice are housed in controlled light conditions (lights  
28 on at 03.00h, lights off at 17.00h). To induce  
29 superovulation, adult female mice are injected with 5  
30 international units of Pregnant Mares Serum  
31 Gonadotropin (Cat. No. 4877, Sigma Chemical Company,  
32 Poole, Dorset, England) in 0.1 ml of distilled water,  
33 at 15.00h followed 46 to 48 hours later by injection of

1 5 international units of Human Chorionic Gonadotropin  
2 (HCG) (Cat. No. CG-10, Sigma Chemical Company, Poole,  
3 Dorset, England) in 0.1 ml of distilled water.  
4 Following HCG injection, the females are housed  
5 individually with mature C57BL/6 X CBA F<sub>1</sub> male mice for  
6 mating. The following morning, mated female mice are  
7 identified by the presence of a vaginal plug.

8  
9 Mated females are killed by cervical dislocation. All  
10 subsequent procedures are performed taking precautions  
11 to avoid bacterial and fungal contamination. Oviducts  
12 are excised and placed in M2 culture medium (Hogan,  
13 Costantini and Lacy "Manipulating the Mouse Embryo: A  
14 Laboratory Manual" Cold Spring Harbor Laboratory (1986)  
15 pp254-256). The fertilised eggs are dissected out of  
16 the ampullae of the oviducts into M2 containing  
17 300 µg/ml hyaluronidase (Type IV-S, Cat. No. H3884,  
18 Sigma Chemical Company, Poole, Dorset, England) to  
19 release the cumulus cells surrounding the fertilised  
20 eggs. Once the eggs are free of cumulus, they are  
21 washed free of hyaluronidase and, until required for  
22 injection, are kept at 37°C either in M2 in a  
23 humidified incubator, or in a drop (100 - 200 µl) of  
24 Medium No. 16 (Hogan, Costantini and Lacy "Manipulating  
25 the Mouse Embryo: A Laboratory Manual" Cold Spring  
26 Harbor Laboratory (1986) pp254-255, and 257), under  
27 mineral oil (Cat. No. 400-5, Sigma Chemical Company,  
28 Poole, Dorset, England) in an atmosphere of 95% air, 5%  
29 CO<sub>2</sub>.

30

31 Injection of DNA

32

33 The DNA to be injected is diluted to approximately

1 1.5  $\mu\text{g}/\text{ml}$  in AnalaR water (Cat. No. 10292 3C, BDH  
2 Chemicals, Burnfield Avenue, Glasgow G46 7TP,  
3 Scotland), previously sterilised by filtration through  
4 a 0.2  $\mu\text{m}$  pore size filter (Cat. No. SM 16534,  
5 Sartorius, 18 Avenue Road, Belmont, Surrey SM2 6JD,  
6 England). All micropipette tips and microcentrifuge  
7 tubes used to handle the DNA and diluent are rinsed in  
8 0.2  $\mu\text{m}$ -filtered water, to remove particulate matter  
9 which could potentially block the injection pipette.  
10 The diluted DNA is centrifuged at 12000 x g for at  
11 least 15 minutes to allow any particulate matter to  
12 sediment or float; a 20  $\mu\text{l}$  aliquot is removed from just  
13 below the surface and used to fill the injection  
14 pipettes.

15  
16 Injection pipettes are prepared on the same day they  
17 are to be used, from 15cm long, 1.0mm outside diameter,  
18 thin wall, borosilicate glass capillaries, with  
19 filament (Cat. No. GC100TF-15; Clark Electromedical  
20 Instruments, PO Box 8, Pangbourne, Reading, RG8 7HU,  
21 England), by using a microelectrode puller (Campden  
22 Instruments, 186 Campden Hill Road, London, England).  
23 DNA (approximately 1  $\mu\text{l}$ ) is introduced into the  
24 injection pipettes at the broad end; it is carried to  
25 the tip by capillary action along the filament. To  
26 prevent evaporation of water from the DNA solution,  
27 approximately 20  $\mu\text{l}$  Fluorinert FC77 (Cat. No. F4758,  
28 Sigma Chemical Company, Poole, Dorset, England) is laid  
29 over the DNA solution. The filled injection pipettes  
30 are stored at 4°C until required.

31  
32 The holding pipette (used to immobilise the eggs for  
33 microinjection) is prepared from 10cm long, 1.0mm

1 outside diameter, borosilicate glass capillaries (Cat.  
2 No. GC100-10; Clark Electromedical Instruments, PO Box  
3 8, Pangbourne, Reading RG8 7HU, England). The glass is  
4 heated over a small flame and pulled by hand to give a  
5 2 - 4 cm long section with a diameter of 80 - 120  $\mu$ m.  
6 Bends are introduced into the pipette, the glass is  
7 broken and the tip is polished using a microforge  
8 (Research Instruments, Kernick Road, Penryn TR10 9DQ,  
9 England).

10

11 A cover slip chamber is constructed in which to  
12 micromanipulate the eggs. The base of the cover-slip  
13 chamber is a 26 x 76 x (1 - 1.2)mm microscope slide  
14 (Cat. No. ML330-12, A and J Beveridge Ltd, 5 Bonnington  
15 Road Lane, Edinburgh EH6 5BP, Scotland) siliconised  
16 with 2% dimethyldichlorosilane (Cat. No. 33164 4V, BDH  
17 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)  
18 according to the manufacturer's instructions; two glass  
19 supports (25 x 3 x 1 mm, cut from microscope slides)  
20 are fixed onto the slide with high vacuum silicone  
21 grease (Cat. No. 33135 3N, BDH Chemicals, Burnfield  
22 Avenue, Glasgow G46 7TP, Scotland) parallel to and  
23 approximately 2mm from the long sides of the slide,  
24 half way along the length of the slide. A further two  
25 glass supports are fixed on top of the first pair, and  
26 the top surface is smeared with silicone grease.  
27 300  $\mu$ l of medium M2 are pipetted into the space between  
28 the supports, and a 22 x 22 mm cover-slip (Cat. No.  
29 ML544-20, A and J Beveridge Ltd, 5 Bonnington Road  
30 Lane, Edinburgh EH6 5BP, Scotland) is lowered onto the  
31 supports, a seal being formed by the grease.  
32 Dow-Corning fluid (50 cs) (Cat. No. 63006 4V, BDH  
33 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)

1 is pipetted into the open ends of the chamber, to cover  
2 the medium.

3  
4 Batches of eggs (30 to 100) are placed into a  
5 cover-slip chamber for manipulation. The chamber is  
6 mounted on the microscope (Diaphot, Nikon (UK) Ltd,  
7 Haybrooke, Telford, Shropshire, England) which has 4x  
8 bright field, 10x phase contrast and 40x differential  
9 interference contrast (DIC) objectives, and 10x  
10 eyepieces. Mechanical micromanipulators (Cat. Nos.  
11 520 137 and 520 138, E. Leitz (Instruments) Ltd, 48  
12 Park Street, Luton, England) are mounted adjacent to  
13 the microscope and are used to control the positions of  
14 the holding and injection pipettes.

15  
16 The holding pipette and DNA-containing injection  
17 pipette are mounted in modified instrument tubes (Cat.  
18 No. 520 145, E. Leitz (Instruments) Ltd, 48 Park  
19 Street, Luton, England) which are in turn mounted onto  
20 the micromanipulators via single unit (Cat. No.  
21 520 142, E. Leitz (Instruments) Ltd, 48 Park Street,  
22 Luton, England) and double unit (Cat. No. 520 143, E.  
23 Leitz (Instruments) Ltd, 48 Park Street, Luton,  
24 England) instrument holders, respectively. The  
25 instrument tubes are modified by gluing onto Clay Adams  
26 "Intramedic" adapters (2.0-3.5 mm tubing to female  
27 Luer, Cat. No. 7543D, Arnold R. Horwell Ltd, 2  
28 Grangeway, Kilburn High Road, London NW6 2BP, England),  
29 which are used to connect the instrument tubes to  
30 approximately 2 metres of polythene tubing (1.57 mm  
31 inside diameter, 2.9 mm outside diameter, Cat. No.  
32 F21852-0062, R.B. Radley & Co, Ltd, London Road,  
33 Sawbridgeworth, Herts CM21 9JH, England), further

1 "Intramedic" adapters are connected to the other ends  
2 of the polythene tubing to facilitate connection to the  
3 syringes used to control the holding and injection  
4 pipettes.

5  
6 Injection is controlled using a 20ml or a 100ml glass  
7 syringe (Cat. Nos. M611/20 and M611/31, Fisons, Bishop  
8 Meadow Road, Loughborough LE11 0RG, England), the  
9 plunger of which is lightly greased with high vacuum  
10 silicone grease (Cat. No. 33135 3N, BDH Chemicals,  
11 Burnfield Avenue, Glasgow G46 7TP, Scotland).

12  
13 Holding of eggs is controlled with an Agla micrometer  
14 syringe (Cat. No. MS01, Wellcome Diagnostics, Temple  
15 Hill, Dartford DA1 5AH, England), which is fitted with  
16 a light spring around the plunger. The Agla syringe is  
17 connected via a 3-way stopcock (Cat. No. SYA-580-L,  
18 Gallenkamp, Belton Road West, Loughborough LE11 0TR,  
19 England), to the "Intramedic" adapter, the third port  
20 of the stopcock is connected to a reservoir of  
21 Fluorinert FC77 (Cat. No. F 4758, Sigma Chemical  
22 Company, Poole, Dorset, England), which fills the Agla  
23 syringe, polythene tubing, instrument tube and holding  
24 pipette.

25  
26 The tip of the injection pipette is broken off against  
27 the holding pipette, to increase the tip diameter to a  
28 size which allows free passage of the DNA solution and  
29 which is small enough to allow injection without lethal  
30 damage to the eggs ( $\leq 1 \mu\text{m}$ ). The flow of DNA through  
31 the pipette tip is checked by viewing under phase  
32 contrast conditions whilst pressure is applied to the  
33 injection syringe (the DNA solution will appear as a  
34 bright plume emerging from the tip of the pipette).



1 One by one, fertilised eggs are picked up on the  
2 holding pipette, and one or both pronuclei brought into  
3 the same focus as the injection pipette (using the 40x  
4 objective and DIC conditions; the correction ring on  
5 the objective is adjusted for optimum resolution). The  
6 injection pipette is inserted into one of the  
7 pronuclei, avoiding the nucleoli, pressure is applied  
8 to the injection syringe and once swelling of the  
9 pronucleus is observed, pressure is released and the  
10 injection pipette is immediately withdrawn. When  
11 pipettes block, the blockage may be cleared by  
12 application of high pressure on the injection syringe  
13 or by breaking off a further portion of the tip. If  
14 the blockage cannot be cleared, or if the pipette tip  
15 becomes dirty, the pipette is replaced.

16  
17 After injection, the eggs are cultured overnight in  
18 medium No. 16 under oil in an atmosphere of 5% CO<sub>2</sub>.  
19 Eggs which cleave to two cells during overnight culture  
20 are implanted into pseudopregnant foster mothers.

21  
22 Random-bred albino (MF1, Harlan Olac Ltd, Shaw's Farm,  
23 Bicester, OX6 OTP, England) female mice are mated with  
24 vasectomised (Hogan, Costantini and Lacy, "Manipulating  
25 the Mouse Embryo: A Laboratory Manual" Cold Spring  
26 Harbor Laboratory (1986); Rafferty, "Methods in  
27 experimental embryology of the mouse", The Johns  
28 Hopkins Press, Baltimore, USA (1970)) MF1 male mice.  
29 The matings are performed one day later than those of  
30 the superovulated egg donors. MF1 females which have a  
31 detectable vaginal plug the following morning are used  
32 as foster mothers. The ideal weight of foster mothers  
33 is 25 to 30g. Each foster mother is anaesthetised by

1 intraperitoneal injection of Hypnorm/Hypnovel (10  $\mu$ l/g  
2 body weight) at 2/3 the concentration recommended by  
3 Flecknell (Veterinary Record, 113, 574) (Hypnorm: Crown  
4 Chemical Co, Ltd, Lamberhurst, Kent TN3 8DJ, England;  
5 Hypnovel: Roche Products Ltd, PO Box 8, Welwyn Garden  
6 City, Herts AL7 3AY, England) and 20 to 30 2-cell eggs  
7 are transferred into one oviduct by the method  
8 described by Hogan, Costantini and Lacy ("Manipulating  
9 the Mouse Embryo: A Laboratory Manual" Cold Spring  
10 Harbor Laboratory (1986)). As an option, to minimise  
11 bleeding from the ovarian bursa, 2  $\mu$ l of 0.01% (w:v)  
12 epinephrine bitartrate (Cat. No. E4375, Sigma Chemical  
13 Company, Poole, Dorset, England) dissolved in distilled  
14 water is applied to the bursa a few minutes before  
15 tearing it. Foster mothers are allowed to deliver  
16 their offspring naturally unless they have not done so  
17 by 19 days after egg transfer, in which case the pups  
18 are delivered by hysterectomy, and are fostered.  
19 Following normal mouse husbandry, the pups are weaned  
20 at 3 to 4 weeks of age and housed with other mice of  
21 the same sex only.

22  
23 Transgenic female mice may be used for the breeding of  
24 subsequent generations of transgenic mice by standard  
25 procedures and/or for the collection of milk and RNA.  
26 Transgenic male mice are used to breed subsequent  
27 generations of transgenic mice by standard procedures.  
28 Transgenic mice of subsequent generations are  
29 identified by analysis of DNA prepared from tails, as  
30 described below.

31  
32  
33

1 SHEEP

2

3 The generation of transgenic sheep is described in  
4 detail in International Patent Application No.  
5 WO-A-8800239 (Pharmaceutical Proteins Ltd) and by  
6 Simons, Wilmut, Clark, Archibald, Bishop & Lathe (1988)  
7 Biotechnology 6, 179-183.

8

9 C. IDENTIFICATION OF TRANSGENIC INDIVIDUALS

10

11 MICE

12

13 When the pups are at least 4 weeks of age, a biopsy of  
14 tail is taken for the preparation of DNA. The pups are  
15 anaesthetised by intraperitoneal injection of  
16 Hypnorm/Hypnovel (10 µl/g body weight) at 1/2 the  
17 concentration recommended by Flecknell (Veterinary  
18 Record, 113, 574). Once anaesthetised, a portion of  
19 tail (1 to 2 cm) is removed by cutting with a scalpel  
20 which has been heated in a Bunsen flame; the hot blade  
21 cauterises the wound and prevents bleeding.

22

23 The tail segments are digested with proteinase  
24 K 200 µg/ml (Sigma) in tail buffer [0.3 M NaAcetate  
25 (not titrated), 10 mM Tris-HCl pH 7.9, 1 mM EDTA pH  
26 8.0, 1% SDS] overnight with shaking at 37°C. The  
27 following day the digests are vortexed briefly to  
28 disaggregate the debris. Aliquots of digested tail are  
29 phenol/chloroform extracted once, chloroform extracted  
30 once and then DNA is recovered by precipitation with an  
31 equal volume of isopropanol.

32

33

1 'Tail DNA' is digested with restriction enzyme(s), and  
2 subjected to agarose gel electrophoresis. The  
3 separated DNA is then 'Southern' blotted to Hybond<sup>TM</sup> N  
4 (Amersham) nylon membranes as described in the Amersham  
5 Handbook 'Membrane transfer and detection methods'  
6 (P1/162/86/8 published by Amersham International plc,  
7 PO Box 16, Amersham, Buckinghamshire HP7 9LL, UK). DNA  
8 bound to the membranes is probed by hybridisation to  
9 appropriate <sup>32</sup>P labelled DNA sequences (eg the  
10 construct DNAs). The DNA probes are labelled with <sup>32</sup>P  
11 by nick-translation as described in 'Molecular Cloning:  
12 a Laboratory Manual' (1982) by Maniatis, Fritsch and  
13 Sambrook, published by Cold Spring Harbor Laboratory,  
14 Box 100, Cold Spring Harbor, USA. Alternatively DNA  
15 probes are labelled using random primers by the method  
16 described by Feinberg and Vogelstein (1984) Analytical  
17 Biochemistry 137, 266-267. Briefly: The plasmid or  
18 phage is cleaved with the appropriate restriction  
19 enzymes and the desired fragment isolated from an  
20 agarose gel. The labelling reaction is carried out at  
21 room temperature by adding the following reagents in  
22 order: H<sub>2</sub>O, 6 µl OLB\*, 1.2 µl BSA, DNA (max. 25 ng),  
23 4 µl <sup>32</sup>P labelled dCTP (PB10205, Amersham plc, Amersham  
24 UK), 1 µl (1 unit) Klenow Polymerase (BCL) to a final  
25 volume of 30 µl.

26

27 \*OLB comprises solution A: 625 µl 2M Tris, pH 8.0 + 25  
28 µl 5M MgCl<sub>2</sub> + 350 µl H<sub>2</sub>O + 18 µl 2-mercaptoethanol  
29 (Sigma); solution B, 2M HEPES (Sigma), titrated to pH  
30 6.6 with NaOH; solution C, Hexa deoxyribonucleotides  
31 (Pharmacia-LKB Biotechnology Cat. No. 27-2166-01). The  
32 labelling reaction is allowed to run overnight and then  
33 the reaction stopped by the addition of 70 µl stop

1 solution (20 mM NaCl, 20 mM Tris pH 7.5, 2mM EDTA,  
2 0.25% SDS, 1  $\mu$ M dCTP). Incorporation is assessed by  
3 TCA precipitation and counting Cerenkov emission.  
4  
5 Hybridisations are carried out in sealed plastic bags  
6 by a modification of the procedure described by Church  
7 and Gilbert (1984). Proceedings of the National  
8 Academy of Sciences (USA) 81, 1991-1995. Briefly: the  
9 probe is used at a concentration of  $1.5 \times 10^6$  Cerenkov  
10 counts/ml of hybridisation buffer (HB: 0.5M sodium  
11 phosphate pH 7.2, 7% SDS, 1mM EDTA). Firstly, the  
12 membrane is prehybridised for 5 minutes in HB (15ml of  
13 buffer per 20 cm<sup>2</sup> membrane) in the plastic bag at 65°C.  
14 The probe is denatured by boiling and added to the same  
15 volume of fresh HB. The plastic bag is cut open and  
16 the prehybridisation solution drained and then the HB +  
17 probe added and the bag re-sealed. The bag and  
18 contents are incubated overnight on a rotary shaker at  
19 65°C. After hybridisation the membrane is washed in 40  
20 mM sodium phosphate, 1% SDS and 1mM EDTA three times  
21 for ten minutes at 65°C and then a final wash is  
22 carried out for 15-30 minutes at this temperature.  
23 Washing is monitored with a hand-held Geiger counter.  
24 The stringency of the washings may be adjusted  
25 according to the particular needs of the experiment.  
26 After the last wash the membrane is blotted dry and  
27 then placed on a dry piece of Whatman filter paper and  
28 wrapped in Saran-wrap. The membrane is exposed to  
29 X-ray film (Agfa CURIX RP-1) using an X-ray cassette at  
30 - 70°C for one or more days.  
31  
32 By comparison with known amounts of construct DNA  
33 treated in the same manner DNA from transgenic

1 individuals can be identified and the number of copies  
2 of the construct DNA which have been integrated into  
3 the genome can be estimated.

4  
5 The same methods are used to identify transgenic  
6 offspring of the founder transgenic individuals.

7  
8 SHEEP

9  
10 The identification of transgenic sheep is described in  
11 detail in International Patent Application No.  
12 WO-A-8800239 (Pharmaceutical Proteins Ltd).

13  
14 D. ANALYSIS OF EXPRESSION - METHODS

15  
16 Collection of Mouse Milk

17  
18 Female mice (at least 7 weeks of age) are housed  
19 individually with adult male mice for mating. After 17  
20 days, the male mice are removed from the cage and the  
21 female mice are observed daily for the birth of  
22 offspring. Milk and/or RNA are collected 11 days after  
23 parturition.

24  
25 For the collection of milk, the pups are separated from  
26 the lactating female mice to allow the build-up of milk  
27 in the mammary glands. After at least 3 hours, 0.3  
28 international units of oxytocin (Sigma, Cat. No.  
29 O 4250) in 0.1 ml of distilled water are administered  
30 by intraperitoneal injection, followed after 10 minutes  
31 by intraperitoneal injection of Hypnorm/Hypnovel  
32 anaesthetic (10 µl/g body weight) at 2/3 the  
33 concentration recommended by Flecknell (Veterinary

1 Record, 113, 574). When fully anaesthetised, the  
2 mammary glands are massaged to expel milk, which is  
3 collected in 50  $\mu$ l capillary tubes (Drummond Microcaps,  
4 Cat. No. PP600-78, A and J Beveridge Ltd, 5 Bonnington  
5 Road Lane, Edinburgh EH6 5BP, Scotland).

6  
7 Mouse milk is diluted 1:5 in distilled water and  
8 centrifuged in an Eppendorf 5415 centrifuge (BDH) to  
9 remove fat. To make whey, 1.0 M HCl was added to give  
10 a final pH of 4.5, thus precipitating the caseins which  
11 were then removed by centrifugation in an Eppendorf  
12 5415 centrifuge. Diluted milk or whey samples were  
13 solubilised by boiling in loading buffer prior to  
14 discontinuous SDS polyacrylamide gel electrophoresis  
15 (Laemmli (1970) Nature 277, 680-684) and immunoblotting  
16 analysis (Khyse-Anderson (1984) Journal of Biochemical  
17 and Biophysical Methods 10, 203-209). Human  
18  $\alpha_1$ -antitrypsin (AAT) was identified on immunoblot  
19 filters by using goat-anti-AT serum [Protein Reference  
20 Unit, Royal Hallamshire Hospital, Sheffield S10 2JF]  
21 and anti-sheep/goat IgG serum conjugated to horseradish  
22 peroxidase [Scottish Antibody Production Unit, Glasgow  
23 and West of Scotland Blood Transfusion Service, Law  
24 Hospital, Carlisle, Lanarkshire ML8 5ES].

25  
26 Amounts of human  $\alpha_1$ -antitrypsin (AAT) in mouse milk  
27 were measured by using LC-Partigen radial  
28 immunodiffusion plates [Behring Diagnostics, Hoescht UK  
29 Ltd, 50 Salisbury Road, Hounslow, Middlesex TW4 6JH].  
30 The radial immunodiffusion (RID) method, which is  
31 designed to detect AAT in body fluids in the  
32 concentration range 8 - 125  $\mu$ g/ml, was carried out  
33 according to the manufacturers instructions. Three

1 dilutions of standard human serum [LC-V, Behring  
2 Diagnostics] were prepared in phosphate buffered saline  
3 (PBS) to give AAT concentrations which fell within the  
4 detection range for the assay.

5  
6 Test milk samples were diluted 1:5 in distilled water  
7 and defatted by spinning briefly in an Eppendorf 5415  
8 centrifuge (BDH). The following control experiment was  
9 carried out in order to assess the effect of the milk  
10 environment on the detection of AAT (the method is  
11 primarily designed for measuring AAT in blood serum).  
12 Milk samples from non-transgenic mice were assayed with  
13 and without defined amounts of added AAT. Samples  
14 (20  $\mu$ l) were loaded into the wells and the plates left  
15 open for 10 - 20 minutes. The plates were then sealed  
16 with the plastic lids provided and left to stand at  
17 room temperature. The diameters of the precipitation  
18 zones were measured after a diffusion time of 2 - 3  
19 days, using a low power binocular microscope fitted  
20 with a lens graticule. At least three independent  
21 readings were recorded and the average measurement (mm)  
22 calculated and squared ( $\text{mm}^2$ ). A calibration curve  
23 plotting zone measurement squared against AAT  
24 concentration was constructed using the values obtained  
25 with the dilutions of standard human serum. This  
26 linear graph was used to calculate the AAT  
27 concentrations in the test samples.

28

#### 29 Preparation of RNA

30

31 RNA may be prepared from mice immediately after milking  
32 or from mice which have not been milked. The lactating  
33 female mouse is killed by cervical dislocation and



1 tissues excised, taking care to avoid cross-  
2 contamination of samples. The procedure is based on  
3 the protocol described by Chirgwin, Przybyla, MacDonald  
4 and Rutter (1979) Biochemistry 18, 5294-5299.

5  
6 The tissue of interest is dissected and placed in 4 ml  
7 of a 4 M solution of Guanadine Thiocyanate in a sterile  
8 30 ml disposable plastic tube. The tissue is  
9 homogenised using an Ultra-Turrax<sup>R</sup> homogeniser at full  
10 speed for 30 - 45 seconds at room temperature. The  
11 homogenate is layered onto a 1.2 ml, 5.7 M CsCl  
12 solution in a 5 ml polyallomer ultracentrifuge tube  
13 (Sorvall Cat. 03127; Du Pont (UK) Ltd, Wedgwood Way,  
14 Stevenage, Hertfordshire SG1 4QN, UK). The RNA is  
15 pelleted through the cushion of CsCl by centrifuging at  
16 36,000 rpm for 12 hrs at 20°C using a Sorvall AH650 or  
17 Beckman SW50.1 swing-out rotor in a Beckman L80  
18 ultracentrifuge (Beckman Instruments (UK) Ltd, Progress  
19 Road, Sands Industrial Estate, High Wycombe, Bucks HP12  
20 4JL, UK). After centrifugation the supernatant is  
21 removed with sterile disposable plastic 5 ml pipettes  
22 and the tube is then very carefully drained. The RNA  
23 which should be visible as an opalescent pellet at the  
24 bottom of the tube is resuspended in 2 ml of 7.5 M  
25 Guanidine Hydrochloride with vigorous vortexing.  
26 Resuspension may take 15 minutes or longer. The  
27 preparation is transferred to a 15 or 30 ml  
28 heat-sterilised Corex<sup>TM</sup> (Du Pont) centrifuge tube and  
29 precipitated by the addition of 50 µl of 1M acetic acid  
30 and 1ml of 100% ethanol and incubation overnight at  
31 -20°C. The RNA is pelleted using a Sorvall SS34 rotor  
32 (Du Pont) in a Sorvall RCB5 refrigerated centrifuge  
33 (Du Pont) at 10,000 rpm for 10 minutes at 2°C. The RNA

1 pellet is resuspended in 2 ml of diethylpyrocarbonate  
2 (Sigma) (DEPC)-treated distilled water by vortexing.  
3 The RNA is re-precipitated by the addition of 600  $\mu$ l of  
4 1M sodium acetate (DEPC-treated) and 3 volumes of 100%  
5 ethanol, resuspended in DEPC treated water and again  
6 precipitated. After the second precipitation from DEPC  
7 water the RNA pellet is resuspended in DEPC water to  
8 the desired final volume (usually 100  $\mu$ l - 500  $\mu$ l).  
9 The concentration of RNA is determined spectro-  
10 photometrically ( $OD_{260nm} = 1$  corresponds to 40  $\mu$ g/ml).  
11 RNA preparations are stored frozen at  $-70^{\circ}\text{C}$ .

12

13 Analysis of RNA

14

15 The expression of the introduced transgene was  
16 investigated in a number of different tissues by  
17 'Northern' blotting of the RNA samples prepared by the  
18 procedure described above. Aliquots (10  $\mu$ g-20  $\mu$ g) of  
19 total RNA were denatured and separated in denaturing  
20 MOPS/formaldehyde (1 - 1.5%) agarose gels and  
21 transferred to Hybond<sup>TM</sup> N (Amersham) nylon membranes as  
22 described in the Amersham Handbook 'Membrane transfer  
23 and detection methods' (PI/162/86/8 published by  
24 Amersham International plc, PO Box 16, Amersham,  
25 Buckinghamshire HP7 9LL, UK). The RNA bound to the  
26 membranes is probed by hybridisation to appropriate  $^{32}\text{P}$   
27 labelled DNA sequences (eg encoding BLG, FIX or AAT).  
28 The labelling and hybridisation procedures are  
29 described in section 1C above.

30

31 In some cases RNA transcripts were detected using an  
32 RNase protection assay. This allows the determination  
33 of the transcriptional start point of the gene. The

1 procedure essentially follows that described by Melton,  
2 Krieg, Rebagliati, Maniatis, Zinn and Green (1984)  
3 Nucleic Acids Research 18, 7035-7054. For example, for  
4 FIX a 145bp SphI-EcoRV fragment from pSltgXSFIX  
5 (WO-A-8800239) which spans the 5' fusion point of BLG  
6 and FIX was cloned into SphI-SmaI cleaved pGEM4  
7 (ProMega Biotec, 2800 South Fish Hatchery Road,  
8 Madison, Wisconsin 53791-9889, USA). A 192 nucleotide  
9 long <sup>32</sup>P labelled, antisense RNA transcript was  
10 generated using SP6 polymerase was used in the RNase  
11 protection assays. After annealing the samples were  
12 digested with RNAase A (BCL) (40 µg/ml) and RNase  
13 T1(BCL) (2 µg/ml) at 37°C for one hour.  
14 Phenol/Chloroform purified samples were electrophoresed  
15 on 8% polyacrylamide/urea sequencing gels.

16

17 EXAMPLE 2: EXPRESSION OF THE AATB CONSTRUCT IN  
18 TRANSGENIC MICE

19

20 The efficient expression of a human plasma protein in  
21 the milk of transgenic mice is exemplified by construct  
22 AATB. The details of the construction of AATB are  
23 given in Example 1. Briefly AATB contains the genomic  
24 sequences for the human (liver) alpha<sub>1</sub>-antitrypsin gene  
25 minus intron 1, fused to the promoter of the ovine  
26 beta-lactoglobulin gene. The fusion point is in the  
27 5'-untranslated region of the BLG gene. It was  
28 anticipated that the presence of the AAT introns would  
29 enhance the levels of expression of the construct. The  
30 large first AAT intron (ca. 5 kb) was omitted in order  
31 to facilitate the DNA manipulation of the construct and  
32 to determine whether all the AAT introns were required  
33 for efficient expression.

1 Unless otherwise stated the analyses of expression are  
2 tabulated. '+' indicates expression as determined by  
3 the presence of the appropriate mRNA transcript  
4 (detected by Northern blotting) or protein (as detected  
5 by radial immunodiffusion (RID) or immunoblotting  
6 (Western blotting)). '-' indicates that the expression  
7 was not detected.

8

9 Transgenic mice carrying the AATB construct

10

11 The AATB construct described in Example 1 was used to  
12 generate transgenic mice by the methods outlined in  
13 Example 1. AATB construct DNA was microinjected into  
14 fertilised mouse eggs on 7 occasions between August  
15 1987 and June 1988. A total of 993 eggs were injected  
16 of which 747 were transferred to recipient  
17 pseudo-pregnant mice. A total of 122 pups were weaned.  
18 Analysis of DNA prepared from tail biopsies, as  
19 described in Example 1C, revealed that of these 122  
20 generation zero (G0) pups 21 carried the AATB construct  
21 as a transgene (see Table 1). These transgenic mice  
22 had between 1 and >20 copies of the AATB construct  
23 integrated into their genome.

24

25 The following policy was adopted for the study of the  
26 expression of the AATB transgene. Where a founder  
27 transgenic G0 individual was male, he was mated to  
28 non-transgenic females to generate G1 offspring. Tail  
29 DNAs from G1 individuals were examined to determine  
30 whether they had inherited the transgene. Female  
31 transgenic G1 mice were used for the analysis of  
32 expression of the AATB transgene by the methods  
33 described in Example 1D. Where a founder transgenic G0

1 individual was female she was used directly for the  
 2 analysis of expression as described in Example 1D. The  
 3 adoption of this policy meant that lines of mice were  
 4 only established where the founder GO animal was male.  
 5 The transmission of the transgenes to subsequent  
 6 generations has also only been determined where the  
 7 founder GO mouse was male. Transmission data for four  
 8 AATB GO males is given in Table 1.

9  
 10 TABLE 1: Mice carrying the AATB construct as a  
 11 transgene.

14	Animal	Sex	Copy	Transmission data	
15	ID		Number	No. of offspring/No. transgenic	
16					
17	AATB15	male	2-5	25	8
18	AATB17	male	10-15	26	16
19	AATB26	male	≥20	34	5
20	AATB28	male	2-5	22	12
21	AATB44	female	15		
22	AATB45	female	1-2		
23	AATB65	female	2-3		
24	AATB69	female	1-2		
25	AATB105	female	20		

26  
 27 Analysis of expression

28  
 29 Fifteen G1 females have been examined for expression of  
 30 the AATB transgene, 8 by protein analysis of milk and 7  
 31 by RNA analysis by the methods described in Example 1.  
 32 A further 5 GO females have been examined by both  
 33 protein analysis of milk and RNA analysis. A total of

1 9 different transgenic mice or mouse-lines were  
2 examined.

3

#### 4 RNA Analysis

5 RNAs isolated from the following tissues were examined  
6 for the presence of AATB transcripts - mammary gland,  
7 liver, kidney, spleen, salivary gland and heart. Total  
8 RNA samples (10  $\mu$ g) from these tissues were analysed by  
9 Northern blotting. A representative Northern blot is  
10 presented as Figure 11 [Lanes 1 & 2, and 3 & 4 contain  
11 mammary (M) and liver (L) samples from control mice;  
12 lanes 5 - 9, AATB26.1 mammary (M), liver (L), kidney  
13 (K), spleen (Sp) and salivary (Sa) RNA samples; lanes  
14 10 - 14, AATB17.3 mammary (M), liver (L), kidney (K),  
15 spleen (Sp) and salivary (Sa) RNA samples. The AAT  
16 transcript of approximately 1400 nucleotides is  
17 arrowed]. The human AAT cDNA probe, p8 $\alpha$ 1ppg,  
18 cross-hybridises with endogenous mouse AAT transcripts  
19 in liver RNA samples. The presence of AAT transcripts  
20 in salivary samples from AATB26.1 and AATB17.3 do not  
21 result from contamination with liver or mammary  
22 material as proved by re-probing the filters with  
23 liver-specific and salivary-specific probes. The  
24 results of this analysis are summarised in Table 2.

25

26

27

28

29

30

31

32

33

1 TABLE 2: Summary of RNA analysis for AATB transgenic  
2 mice.

3	4 Animal ID	5 Generation	6 Tissue (presence/absence of AATB transcripts)					
			Mam.	Liver	Kid.	Spl.	Saliv.	Heart
7	AATB15.2	G1	+	*	?	-	-	-
8	AATB15.13	G1	-	?	-	-	-	NT
9	AATB17.3	G1	+	?	-	-	+	NT
10	AATB17.20	G1	+	-	-	-	+	NT
11	AATB26.1	G1	-	-	-	-	+	NT
12	AATB26.28	G1	-	?	-	-	+	-
13	AATB28.3	G1	-	?	-	-	-	NT
14	AATB28.21	G1	-	?	-	-	-	NT
15	AATB44	GO	+	?	-	-	-	-
16	AATB45	GO	+	?	-	-	-	-
17	AATB65	GO	+	?	-	-	-	-
18	AATB69	GO	+	?	-	-	-	-
19	AATB105	GO	-	?	-	-	+	-

20  
21 [Mam. = mammary gland; Kid. = kidney; Spl. = spleen;  
22 Saliv. = salivary gland; nd = not detected; NT = not  
23 tested]

24 \* presence only detected in poly A+ RNA

25 ? background from endogenous mouse AAT transcripts in  
26 liver precluded an unambiguous determination of whether  
27 there were AATB transcripts present.

28  
29 In order to confirm that the transcripts observed were  
30 being initiated at the beta-lactoglobulin start site in  
31 the AATB constructs, RNAs isolated from the mammary  
32 gland of mouse AATB17.20 and from the salivary gland of  
33 mouse AATB26.1 were examined by an RNase protection

1 assay as described in Example 1D. RNAs isolated from  
2 the liver (AATB17.20 & AATB26.1) and from the mammary  
3 gland (AATB26.1) of these mice were also examined by  
4 RNase protection, as were RNAs from non-transgenic  
5 liver, mammary gland and salivary gland. The  
6 anti-sense probe was produced by transcribing a pGEM  
7 vector (Promega Biotec, 2800 South Fish Hatchery Road,  
8 Madison, Wisconsin 53791-9889) containing a 155 bp SphI  
9 - BamHI fragment derived from the 5' end of the AATA  
10 construct. This 155 bp fragment is identical to that  
11 found in AATB (see pIII-ISpB, Example 1A). Annealing  
12 was carried out under standard conditions and the  
13 hydrolysis of single-stranded RNA performed with RNaseA  
14 and RNaseT1(BCL). A sense transcript was also  
15 transcribed and various amounts of this transcript  
16 included along with 20  $\mu$ g samples of control RNA to  
17 provide an estimation of steady state mRNA levels. A  
18 representative RNase protection gel is shown in Figure  
19 12 [Lanes 1 & 2, AATB17.20 20  $\mu$ g and 10  $\mu$ g total  
20 mammary RNA; lanes 3, 4, 5 & 6, 1000 pg, 200 pg, 100 pg  
21 & 50 pg of control sense transcript; lanes 7 & 8,  
22 AATB26.1 20  $\mu$ g & 10  $\mu$ g total salivary RNA; lanes 9, 10  
23 & 11, 5  $\mu$ g aliquots of mammary polyA+ RNA from  
24 AATB15.2, AATA5.20 and AATA31; lane M HaeIII digested  
25  $\phi$ X174 DNA marker track]. The RNase protection assay  
26 confirmed that the beta-lactoglobulin transcription  
27 start site was being used as predicted in the mammary  
28 tissue of line AATB17 and in the salivary tissue of  
29 line AATB26. The absence of AATB transcripts in the  
30 liver of AATB17.20 and in the liver and mammary gland  
31 of AATB26.1 were also confirmed by RNase protection  
32 assays.

33



1 Protein analysis of milk  
2 Milk samples from 8 G1 females and from 5 G0 females  
3 were assayed for the presence of human  
4  $\alpha_1$ -antitrypsin by the immunoblotting methods  
5 described in Example 1D. The results of this analysis  
6 are summarised in Table 3. A representative immunoblot  
7 of diluted milk samples from transgenic and normal mice  
8 is shown as Figure 13 [lanes 1, pooled human serum; 2,  
9 control mouse milk; 3, AATB 15.10 milk; 4, AATB 17.24  
10 milk; 5, AATB 17.23 milk; 6, AATB 15.20 milk; 7,  
11 control mouse milk; 8 & 9, marker proteins]. The human  
12 AAT (arrowed) is clearly evident in preparations from  
13 mice AATB17.23 and AATB17.24 and just about visible in  
14 milk from mouse AATB15.10]. Cross reaction of the  
15 anti-human sera to endogenous mouse AAT (which migrates  
16 slightly faster than its human counterpart) is also  
17 evident.

18

19 Amounts of human  $\alpha_1$ -antitrypsin in transgenic mouse  
20 milk were estimated using LC-Partigen radial  
21 immunodiffusion plates [RID] [Behring Diagnostics,  
22 Hoescht UK Ltd, 50 Salisbury Road, Hounslow, Middlesex  
23 TW4 6JH] as described in Example 1D (see Table 3).  
24 Normal mouse milk samples with and without human  
25  $\alpha_1$ -antitrypsin were included as controls.

26

27

28

29

30

31

32

33

1 TABLE 3

2	3	4	5	6
	Animal	Generation	Immunoblot	RID
	ID		presence/absence	protein mg/ml
6	AATB15.10	G1	+	NT
7	AATB15.20	G1	-	NT
8	AATB17.23	G1	+	0.448
9	AATB17.24	G1	+	0.533
10	AATB26.14	G1	-	NT
11	AATB26.28	G1	-	NT
12	AATB28.11	G1	-	NT
13	AATB28.14	G1	-	NT
14	AATB44	GO	+	0.87
15	AATB45	GO	+	0.088
16	AATB65	GO	+	0.091
17	AATB69	GO	+	0.465
18	AATB105	GO	-	-

19

20 [NT = not tested]

21

22 Of the nine different AATB transgenic mice or  
 23 mouse-lines examined, five efficiently directed  
 24 expression of human  $\alpha_1$ -antitrypsin in milk. A  
 25 sixth line (AATB15) also exhibited mammary expression,  
 26 but at lower levels. This analysis proves that the  
 27 AATB construct contains sufficient information to  
 28 direct efficient expression of human  $\alpha_1$ -antitrypsin  
 29 in the mammary glands of transgenic mice. There  
 30 appears to be some relaxation of the tissue-specificity  
 31 of the BLG promoter such as to allow it to function in  
 32 salivary gland as well as in the mammary gland. The  
 33 first intron of the AAT gene is not necessary for

1 efficient expression of the hybrid gene AATB. The  
2 introns and 3' flanking sequences of the BLG gene are  
3 evidently not essential for efficient mammary gland  
4 expression from the BLG promoter. The 5' flanking  
5 sequences of the BLG gene from SalI through SphI to the  
6 PvuII site in the 5'-untranslated of the BLG gene are  
7 sufficient to direct the efficient mammary expression  
8 of a heterologous gene as exemplified by AAT.

9

10 EXAMPLE 3 : COMPARATIVE EXPRESSION OF BLG CONSTRUCTS

11

12 The efficient expression of a human plasma protein in  
13 the milk of transgenic mice is exemplified by construct  
14 AATB. In this section the expression analyses of  
15 different constructs encoding a human plasma protein,  
16 either FIX or AAT, are given. The details of their  
17 constructions are given in Example 1A. Expression  
18 analyses of two configurations of the BLG gene are also  
19 given and serve to further define the BLG sequences  
20 that may be required for expression in the mammary  
21 gland. Unless otherwise stated the analyses of  
22 expression are tabulated. '+' indicates expression as  
23 determined by the presence of the appropriate mRNA  
24 transcript (detected by Northern blotting) or protein  
25 (as detected by radioimmunoassay (RIA), radial-  
26 immunodiffusion (RID), Coomassie blue staining or  
27 Western blotting. '-' indicates that expression was  
28 not detected.

29

30 **FIXA:**

31

32 Construction and expression of this construct is  
33 described in detail in WO-A-8800239 (designated

1 pSS1tgXS-FIX or pSS1tgXS-TARG). It comprises cDNA  
 2 sequences encoding human blood clotting factor IX (FIX)  
 3 inserted into the first exon of the BLG gene.  
 4 Transgenic sheep have been produced which carry this  
 5 construct and these have been analysed for the  
 6 expression of human FIX by Northern blotting of mammary  
 7 RNA and radioimmunoassays of milk:-

8	9	10	11	12	13
	Sheep	Description	RNA	FIX Protein (iu*/l)	
10	6LL240	GO female	+	+: 4.7 <sup>a</sup> , 8.0 <sup>b</sup>	
11	6LL231	GO female	+	+: 4.0a, 4.3b	
12	7R45	G1 female@	+	+: / 5.7b	
13	7R39	G1 female@	+	+: / 6.4b	

14  
 15 [a, analysis by RIA in 1987; b, analysis in 1988;  
 16 \*, 1 iu = 5 µg; @, daughters of transgenic male 6LL225]

17  
 18 The human FIX protein in transgenic sheep milk has been  
 19 visualised by Western blotting and also shown to have  
 20 biological activity. However, the level of protein in  
 21 the milk is far below that necessary for commercial  
 22 exploitation.

#### 23 24 AATA:

25  
 26 This construct comprises the cDNA encoding human AAT  
 27 inserted into the first exon of the BLG gene. It is  
 28 equivalent to FIXA and thus can be considered as an  
 29 example of the generalised construct designated  
 30 pSS1tgXS-TARG as described in WO-A-8800239. It has  
 31 been used to produce transgenic sheep and mice.

32  
 33

1	Sheep	Description	RNA	AAT Protein*
2	6LL273	GO female	-	-
3	6LL167	GO female	nd	+ (2-10 µg/ml)
4	7LL183	GO female	nd	nd
5	*protein detected and estimated by Western blotting of			
6	milk samples			
7	nd; not done			

8  
9 Western blots of milk whey samples from normal and the  
10 two transgenic sheep analysed are shown in Figure 14  
11 [lanes 1, 7LL167(AATA); 2, control sheep whey; 3, human  
12 serum pool; 4, 7LL167(AATA); 5, 6LL273(AATA); 6,  
13 control sheep whey].

14  
15 The human AAT (arrowed) is clearly evident in milk whey  
16 samples from 6LL167 but is not present in that from  
17 6LL273 or control sheep milk. Under these conditions  
18 endogenous AAT present in sheep milk is detected by the  
19 anti-human sera and has a greater electrophoretic  
20 mobility than its human counterpart.

21  
22 The levels of human AAT estimated to be present in the  
23 transgenic sheep milk are low and are not sufficient  
24 for commercial exploitation.

25  
26 Expression of the AATA construct has also been studied  
27 in transgenic mice.

28  
29  
30  
31  
32  
33

1	Mice	Description	RNA	AAT protein*
2	AATA1.5	line segregating	-	-
3		from AATA1		
4	AATA1.8	line segregating		
5		from AATA1	+	+ (<<2 $\mu$ g/ml)
6	AATA5	mouse-line	+	+ (2-10 $\mu$ g/ml)
7	AATA31	mouse-line	-	-

8 \*AAT protein detected and estimated by Western  
9 blotting.

10

11 Western blots of TCA precipitated whey samples from  
12 normal and transgenic mice are shown in Figure 15  
13 [Lanes 1, human  $\alpha_1$ -antitrypsin antigen (Sigma); 2,  
14 human serum; 3, mouse serum; 4, AATA 1.8.1 whey; 5,  
15 AATA 1.5.10 whey; 6, human and mouse serum; 7, control  
16 mouse whey; 8, AATA 5.30 whey; 9, AATA 1 whey; 10,  
17 human serum; 11, mouse serum]. The human AAT (arrowed)  
18 is clearly evident in preparations from mouse-line  
19 AATA5 and is just about visible in mouse-line AATA1.8.  
20 Cross-reaction of the anti-human sera with endogenous  
21 mouse AAT (which migrates slightly faster than its  
22 human counterpart) is also evident.

23

24 The levels of expression observed in mouse-line AATA5  
25 are of the same order of magnitude as is observed in  
26 transgenic sheep 7LL167, and as such would not prove  
27 commercial even if obtained in a dairy animal such as a  
28 sheep.

29

30 BLG-BLG

31

32 This construct comprises the BLG cDNA inserted into  
33 exon1 of the BLG structural gene. The construct is

1 analogous to AATA and FIXA (ie pSS1tgXS-TARG) in that  
 2 the complete structural gene of BLG is present as well  
 3 as the cDNA insert. In this case, however, the insert  
 4 is a cDNA encoding a milk protein, rather than a cDNA  
 5 from a gene normally expressed in another tissue. The  
 6 expression of this construct was assessed in transgenic  
 7 mice.

9 Mice	Description	RNA	BLG protein*
10 BB4	GO female	+	+(<.005mg/ml)
11 BB5	GO female	+	+(<.005mg/ml)
12 BB19	GO female	+	+(<.005mg/ml)
13 BB47	GO female	+	+(<.005mg/ml)
14 BB55	GO female	nd	+(<.005mg/ml)

15 \*detected and estimated by Western blotting

16 nd = not determined

17  
 18 The construct was expressed tissue-specifically in the  
 19 four mice in which RNA was analysed. In all five  
 20 animals low levels of BLG were detected in the milk.  
 21 These levels of BLG are far below that observed with  
 22 expression of the normal structural BLG gene (eg see  
 23 Example 7 in WO-A-8800239). The data show that the  
 24 'A-type' construct even when encoding a natural milk  
 25 protein gene such as BLG (which is known to be capable  
 26 of very high levels of expression in the mammary gland)  
 27 is not expressed efficiently in the mammary gland of  
 28 transgenic mice. This suggests that it may be the  
 29 configuration of cDNA (whether FIX, AAT or BLG) with  
 30 the genomic BLG sequence (ie insertion into the first  
 31 exon) which is responsible for the low levels of  
 32 expression of this type of construct.

33

1    **AATD**

2

3    This construct comprises the AAT cDNA fused to 5' BLG  
4    sequences and with 3' sequences from exons 6 and 7 of  
5    BLG and the 3' flanking sequences of the BLG gene.  
6    This gene contains no introns.    Its potential for  
7    expression was assessed in transgenic mice:-

8

9	Mice	Description	RNA	AAT Protein*
10	AATD12	GO female	-	-
11	AATD14	GO female	-	-
12	AATD31	GO female	-	-
13	AATD33	GO female	-	-
14	AATD9	mouse-line	-	-
15	AAT21	mouse-line	-	-
16	AATD41	mouse-line	-	-
17	AATD47	mouse-line	-	-

18    \*assessed by Western blotting

19

20    None of the transgenic mice carrying AATD expressed the  
21    transgene.

22

23    **FIXD**    This is an analogous construct to AATD and  
24    comprises the FIX cDNA sequences fused to BLG 5' and 3'  
25    sequences (including exons 6 and 7) and contains no  
26    introns.    Expression was assessed in transgenic mice.

27

28

29

30

31

32

33



1	Mice	Description	RNA	FIX Protein*
2	FIXD11	GO female	-	-
3	FIXD14	GO female	-	-
4	FIXD15	GO female	-	-
5	FIXD16	GO female	-	-
6	FIXD18	GO female	-	-
7	FIXD20	mouse-line	-	-
8	FIXD23	mouse-line	-	-
9	FIXD24	mouse-line	-	-
10	FIXD26	mouse-line	-	-

11 \*assessed by Western blotting

12

13 None of the transgenic mice carrying FIXD expressed the  
14 transgene.

15

16 These data, together with those from AATD, suggest that  
17 a simple configuration of BLG 5' and 3' sequences and  
18 target cDNA sequences (ie FIX or AAT) in which no  
19 introns are present in the construct will not be  
20 expressed efficiently, if at all, in the mammary gland  
21 of a transgenic animal.

22

#### 23 AATC

24

25 This construct comprises the AAT cDNA inserted into the  
26 second exon of BLG. It was constructed to determine  
27 whether or not inserting the target cDNA (in this case  
28 AAT) at a site distant from the promoter (ie in the  
29 second rather than in the first exon) would improve the  
30 levels of expression. Expression was assessed in  
31 transgenic mice.

32

33

1	Mice	Description	RNA	AAT Protein*
2	AATC14	GO female	-	-
3	AATC24	GO female	-	-
4	AATC25	GO female	-	-
5	AATC30	GO female	-	-
6	AATC4	mouse-line	+	-
7	AATC5	mouse-line	-	-
8	AATC27	mouse-line	-	-

9 \*assessed by Western blotting

10

11 Only one out of seven 'lines' expressed the transgene  
12 as determined by RNA; in this line no AAT protein was  
13 detected, presumably because re-initiation from the  
14 initiating ATG of the AAT sequences did not occur. In  
15 the RNA-expressing line expression appeared to occur  
16 only in the mammary gland although at low levels.  
17 These data would suggest that moving the site of  
18 insertion of the target cDNA to the second exon (and  
19 thus including intron 1 of the BLG) does not yield  
20 improved levels of expression of the target cDNA (in  
21 this case AAT).

22

## 23 DELTA A2

24

25 This construct contains the minimum ovine BLG sequences  
26 that have so far been shown in transgenic mice to be  
27 required for efficient and tissue-specific expression  
28 of BLG in the mammary gland. It is a 5' deletion  
29 derivative of pSS1tgXS (WO-A-8800239) and has only  
30 799 bp of sequence flanking the published mRNA cap site  
31 (Ali and Clark, (1988) J. Mol. Biol. 199, 415-426).  
32 This deleted version of pSS1tgXS has been used to  
33 produce transgenic mice.

1	Mouse	Description	RNA	BLG Protein*
2	DELTA A2/1	GO female	+	+ ~2mg/ml
3	DELTA A2/28	GO female	+	+ ~3mg/ml
4	DELTA A2/38	GO female	+	+ <0.15mg/ml

5

6 Detected by Coomassie blue staining: estimated  
7 densitometrically.

8

9 The DELTA A2 constructs shows that 799 bp of 5'  
10 flanking sequences are sufficient for correct and  
11 efficient expression of BLG in the mammary gland of  
12 transgenic mice. This construct also contains the  
13 4.9kb transcription unit of BLG and 1.9kb of 3'flanking  
14 sequences. It is conceivable that important regulatory  
15 sequences for mammary expression are present in these  
16 regions. (However, note the result with AATB in which  
17 these sequences were absent and yet efficient mammary  
18 expression was obtained.)

19

#### 20 EXAMPLE 4 : PREPARATION OF FACTOR IX CONSTRUCT

21

##### 22 Strategy

23

24 The expression in transgenic sheep of a human Factor IX  
25 gene, called BLG-FIX, is disclosed in WO-A-8800239 and  
26 Clark et al (1989) (Biotechnology, 7 487-492), both of  
27 which are herein incorporated by reference, insofar as  
28 the law allows. Since this construct has been  
29 previously referred to as FIX A, this nomenclature is  
30 retained. Essentially the FIX A construct comprises  
31 the insertion of a human FIX cDNA into the first intron  
32 of the complete (ie all exons and introns present)  
33 sheep betalactoglobulin (BLG) gene. This example

1 relates to the modification of this FIX A construct to  
2 the effect that the first intron of the human genomic  
3 FIX gene has been inserted at the appropriate position,  
4 into the FIX cDNA, so that on transcription of the new  
5 gene, a primary transcript containing an intron will be  
6 produced. When this transcript is correctly spliced, a  
7 transcript will be generated, which on translation,  
8 will generate exactly the same protein as the original  
9 FIX A construct.

10

11 The construction route shown below is complicated, but  
12 the methods used are as described in Example 1. The  
13 difficulties were caused by the size of human FIX  
14 genomic DNA fragments and the requirement to develop  
15 new shuttle vectors to allow the suitable manipulation  
16 of the BLG and FIX DNA sequences.

17

18 A.

19 Aims

20 Construction of -

21

- 22 a) pUC PM - modified cloning vector.
- 23 b) pUC XS - pUC PM containing BLG genomic DNA.
- 24 c) pUC XS/RV - pUC XS containing a unique EcoRV  
25 restriction site in the BLG 5'  
26 untranslated region.

27

28 Details

29

- 30 i A double stranded synthetic linker DNA including  
31 in the following order the restriction sites for  
32 the enzymes EcoRI, PvuI, MluI, SalI, EcoRV, XbaI,  
33 PvuI, MluI, HindIII (see Fig 16a) was ligated into

- 1        EcoRI/HindIII digested, gel purified, pUC 18  
2        (Boehringer) to generate pUC PM (see Fig 16a).  
3        The insertion was checked by both restriction  
4        analysis and direct sequencing.  
5  
6        ii    A SalI-XbaI fragment purified from pSS1tgXS (this  
7        contains the XbaI-SalI BLG genomic fragment in  
8        pPOLY III.I (see Figure 3 of WO-A-8800239) was  
9        ligated into SalI/XbaI digested, CIP (calf  
10       intestinal phosphatase) (see Fig 16a) - treated,  
11       gel purified, pUC PM to give pUC XS. This was  
12       checked by restriction analysis.  
13  
14       iii   A synthetic EcoRV linker  
15  
16       (5' TCGACGCGGCCGCGATATCCATGGATCT        )  
17       (       GCTGCGCCGGCGCTATAGGTACCTAGAGATC 5')  
18  
19       was ligated into the unique PvuII site of  
20       PvuII-digested pSS1tgSE (see WO-A-8800239 -  
21       pSS1tgSE comprises a SphI-EcoRI fragment of BLG  
22       inserted into pPOLY III.I; the PvuII site is 30  
23       bases downstream of cap site in the first exon of  
24       BLG) - see Fig 16b.  
25  
26       iv    The SphI-NotI fragment containing the EcoRV linker  
27       was gel purified from pSS1tgSE/RV and ligated into  
28       the SphI, NotI digested, CIP - treated, gel  
29       purified pUC XS, generating pUC XS/RV - see Fig  
30       16b.  
31  
32       This was checked by restriction analysis.  
33

1 B.

2 Aims

3 Construction of -

4  
5 a) Clones 9-3, B6 and 9 H11 - cloning vehicles from  
6 transfer of various portions of FIX genomic DNA.

7  
8 b) Clone 11-6, this comprises exons 1, 2, 3 and  
9 introns 1, 2 of FIX inserted into pUC 9.

10

11 Details

12

13 i Cosmid clone cIX2, containing part of FIX gene,  
14 was obtained from G. Brownlee (see GB-B-2125409,  
15 also P.R. Winslip, D. Phil Thesis, Oxford, and  
16 Anson et al (1988) EMBO J. 7 2795-2799).

17

18 Note In the following description - the assignment of a  
19 base number to a restriction site refers to the  
20 number of bases the site is upstream (mins sign)  
21 or downstream of the cap site in the first FIX  
22 exon. These numbers are obtained by analogy, from  
23 the published FIX sequence of Yoshitake et al  
24 (1985) Biochemistry 24 3736-3750.

25

26 ii Clone 9-3 was produced by ligating gel purified  
27 BamHI (-2032) - EcoRI (5740) fragment from cIX2  
28 into BamHI/EcoRI-digested, CIP-treated, gel  
29 purified, pUC 9 (see Fig 17).

30

31 iii Clone 9 H11 was made by ligating the gel purified  
32 HindIII (810) - HindIII (8329) fragment from cIX2  
33 into HindIII-digested, CIP-treated, gel purified  
34 pUC 9 (see Fig 17).

1    iv    Clone 9-3 was digested with BamHI and HpaI, end  
2           filled with the Klenow enzyme, and the large  
3           fragment was gel purified and ligated to produce  
4           clone B6 (see Fig 17). The net effect of this is  
5           to remove the FIX sequence between -2032 and -830.

6  
7    v    Clone 9H 11 was digested with SalI and BglII,  
8           CIP-treated and then the large fragment, now  
9           lacking the regions between the vector SalI site  
10          and the FIX BglII site (3996) was gel purified.  
11          This was ligated with the gel purified SalI  
12          (vector) - BglII (3996) fragment from clone B6, to  
13          generate clone 11-6 (see Fig 17) which contains  
14          FIX sequence -830 - -8329 (ie exons 1,2,3 introns  
15          1,2).

16  
17    C.

18    Aims

19    Construction of -

- 20  
21    a)    Clone C8 (incorporating 5' portion of FIX cDNA).  
22    b)    Clone C81.SK (incorporating 5' portion of FIX cDNA  
23          + FIX intron I).

24  
25    Details

26  
27    i    FIX A (FIX cDNA in BLG gene, called BLG FIX in  
28          Clark et al, (1989) Biotechnology 7 487-492, also  
29          see WO-A-8800239) was digested with Sph 1/Bst Y 1.  
30          The small fragment was gel purified and ligated  
31          into SphI/BamHI-digested, CIP-treated, pUC 18  
32          (Boehringer) generating clone C8 (see Fig 18) DNA  
33          was prepared by growth in a dam<sup>-</sup> E. coli host (SK  
34          383) to allow Bcl digestion.

1 Note C8 contains most of FIX cDNA and 2 out of 3 BclI  
2 sites (at positions 2 and 81 upstream of the first  
3 nucleotide of the first AUG of the FIX cDNA  
4 sequence shown in Fig 9, GB-B-2125409; these are  
5 equivalent to Bcl sites 46 (exon 1) and 6333 (exon  
6 2) of genomic DNA.

7  
8 ii C8 was digested with BclI, CIP-treated and the  
9 large fragment retained after gel purification.

10  
11 iii Clone 11-6 DNA was prepared from E. coli host SK  
12 383 ( $\text{dam}^-$ ) and the 6287 bp BclI fragment  
13 containing intron 1 purified and ligated with the  
14 large C8 fragment described in ii above, to  
15 generate C81 SK - see Fig 18. The Bcl junctions  
16 were sequenced to confirm reconstruction of Bcl  
17 sites.

18

19 4.

20 Aims

21 Construction of -

22

- 23 a) J FIX A (FIX A insert transferred to pUC PM).  
24 b) SP FIX (A cloning vehicle for transfer of intron 1  
25 to J FIX A).

26

27 Details

28

- 29 i SphI-NotI fragment from FIX A, containing FIX cDNA  
30 and flanking BLG sequence was gel purified and  
31 ligated into SphI/NotI digested, CIP-treated, gel  
32 purified pUC XS/RV to generate J FIX A (see Fig  
33 19).



1    ii    Sph-NruI fragment containing FIX cDNA from J FIX A  
2           was gel purified and ligated into SphI/EcoRV  
3           digested, CIP treated, pSP 72 (promega Biotech) to  
4           generate SP FIX (see Fig 19).

5

6    E.

7    Aims

8    Construction of -

9

- 10   a)    b 11 - cloning vehicle containing FIX intron 1.  
11   b)    J FIX A 1 - final "minigene" construct for  
12           construction of transgenics.

13

14   Details

15

16   i    SP FIX and C81.SK digested to completion with  
17        SphI, then partially digested with Ssp 1\*. A 7.2  
18        kb fragment from C81.SK containing FIX intron 1  
19        was ligated with the CIP-treated, gel purified  
20        large fragment of SP FIX to generate clone b 11  
21        (see Fig 20) which contains the complete FIX cDNA  
22        and FIX intron 1.

23

24   ii   The SphI-NotI fragment from b11 containing the FIX  
25        sequences was gel purified and ligated into  
26        SphI/NotI digested, CIP-treated J FIX A to  
27        generate J FIX A 1 (see Fig 20).

28

29   \*Note - In SP FIX, there is a SspI site in vector which  
30        was not excised in the partially digested fragment  
31        shown. Likewise in C81.SK there are four SspI  
32        sites in the FIX intron. The 7.2K fragment  
33        contains all these four sites and in fact

1 terminates at the SspI site at position 30830 b of  
2 the genomic FIX sequence.

3

4 F.

5

6 Transgenic mice were constructed as described in  
7 Example 1B, and identified as described in Example 1C.  
8 One male and one female transgenic mice were initially  
9 identified.

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

1    CLAIMS

2

3    1.    A genetic construct comprising a 5' flanking  
4    sequence from a mammalian milk protein gene and DNA  
5    coding for a heterologous protein other than the milk  
6    protein, wherein the protein-coding DNA comprises at  
7    least one, but not all, of the introns naturally  
8    occurring in a gene coding for the heterologous protein  
9    and wherein the 5'-flanking sequence is sufficient to  
10   drive expression of the heterologous protein.

11

12   2.    A construct as claimed in claim 1, wherein the  
13   milk protein gene is a beta-lactoglobulin gene.

14

15   3.    A construct as claimed in claim 2, including about  
16   800 base pairs upstream of the beta-lactoglobulin  
17   transcription start site.

18

19   4.    A construct as claimed in claim 2, including about  
20   4.2 kilobase pairs upstream of the beta-lactoglobulin  
21   transcription start site.

22

23   5.    A construct as claimed in claim 1, wherein the  
24   heterologous protein is a serine protease.

25

26   6.    A construct as claimed in claim 2, wherein the  
27   heterologous protein is a blood factor.

28

29   7.    A construct as claimed in claim 1, in which all  
30   but one of the natural introns are present.

31

32   8.    A construct as claimed in claim 1, in which only  
33   one of the natural introns are present.

1 9. A construct as claimed in claim 1 comprising a  
2 3'-sequence.

3

4 10. A method for producing a substance comprising a  
5 polypeptide, the method comprising introducing a DNA  
6 construct as claimed in claim 1 into the genome of an  
7 animal in such a way that the protein-coding DNA is  
8 expressed in a secretory gland of the animal.

9

10 11. A method as claimed in claim 10, wherein the  
11 animal is a mammal and the secretory gland is a mammary  
12 gland.

13

14 12. A vector comprising a genetic construct as claimed  
15 in claim 1.

16

17 13. A cell containing a vector as claimed in claim 12.

18

19 14. An animal cell comprising a construct as claimed  
20 in claim 1.

21

22 15. A transgenic animal comprising a genetic construct  
23 as claimed in claim 1 integrated into its genome.

24

25 16. A transgenic animal as claimed in claim 15 which  
26 is capable of transmitting the construct to its  
27 progeny.

28

29 17. A method for producing a substance comprising a  
30 polypeptide, the method comprising harvesting the  
31 substance from a transgenic animal as claimed in claim  
32 15.

33

1/21

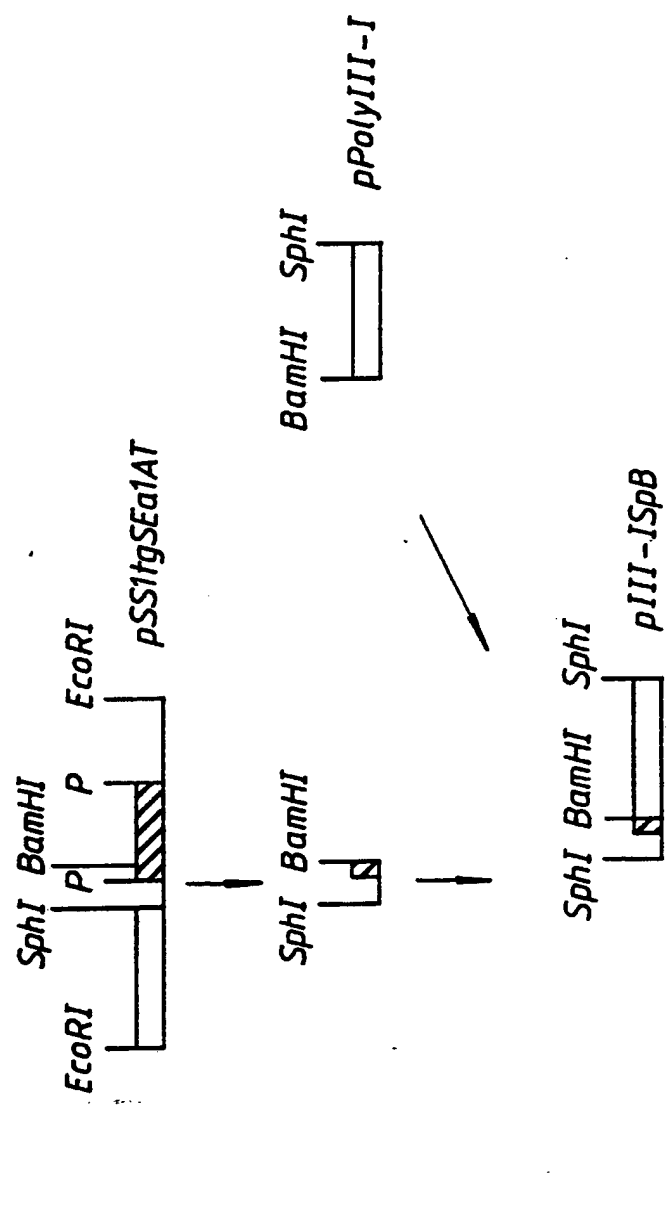


Fig.1.

2/21

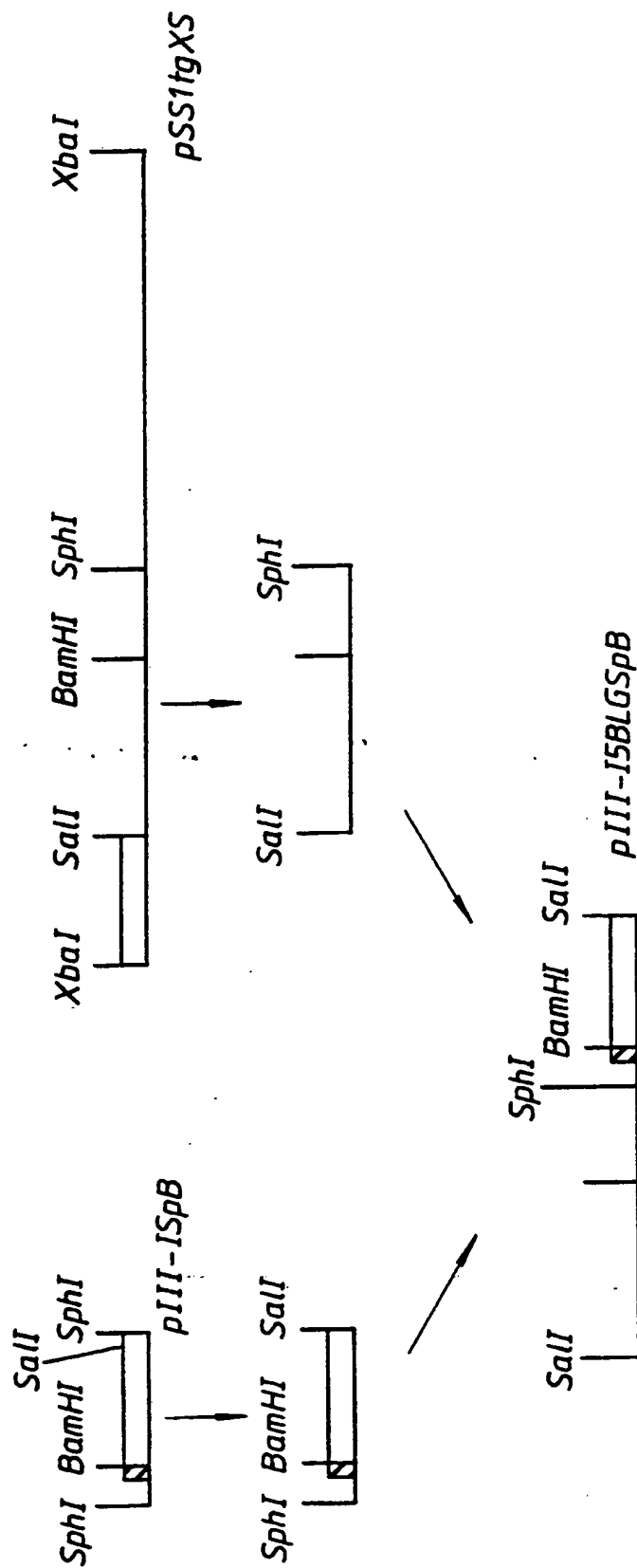



Fig.2.

 alpha1-antitrypsin cDNA sequences

 plasmid sequences

 beta-lactoglobulin sequences

note: not all BamHI sites are shown for pSS1tgXS

3/21

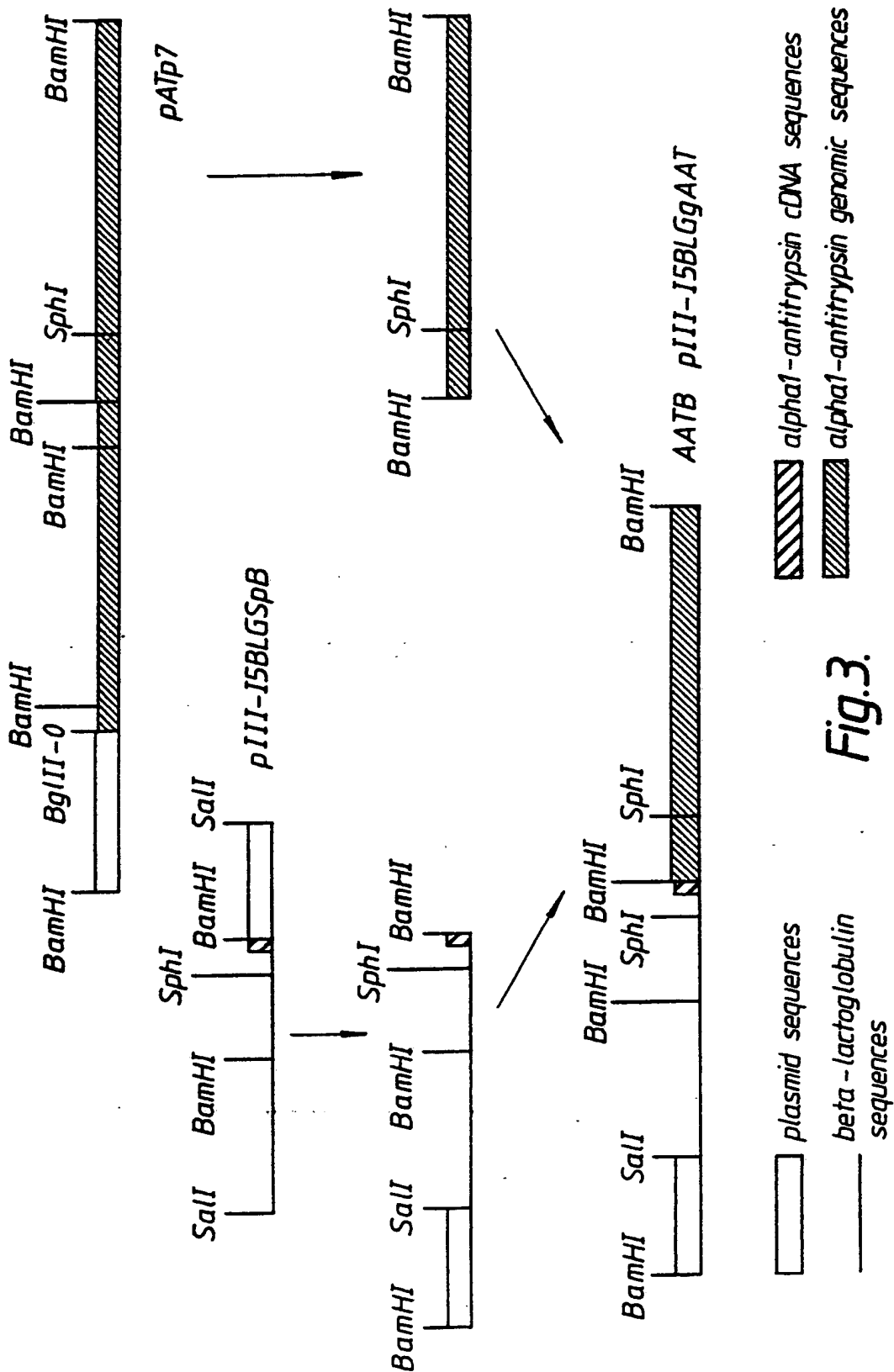


Fig.3.

4/21

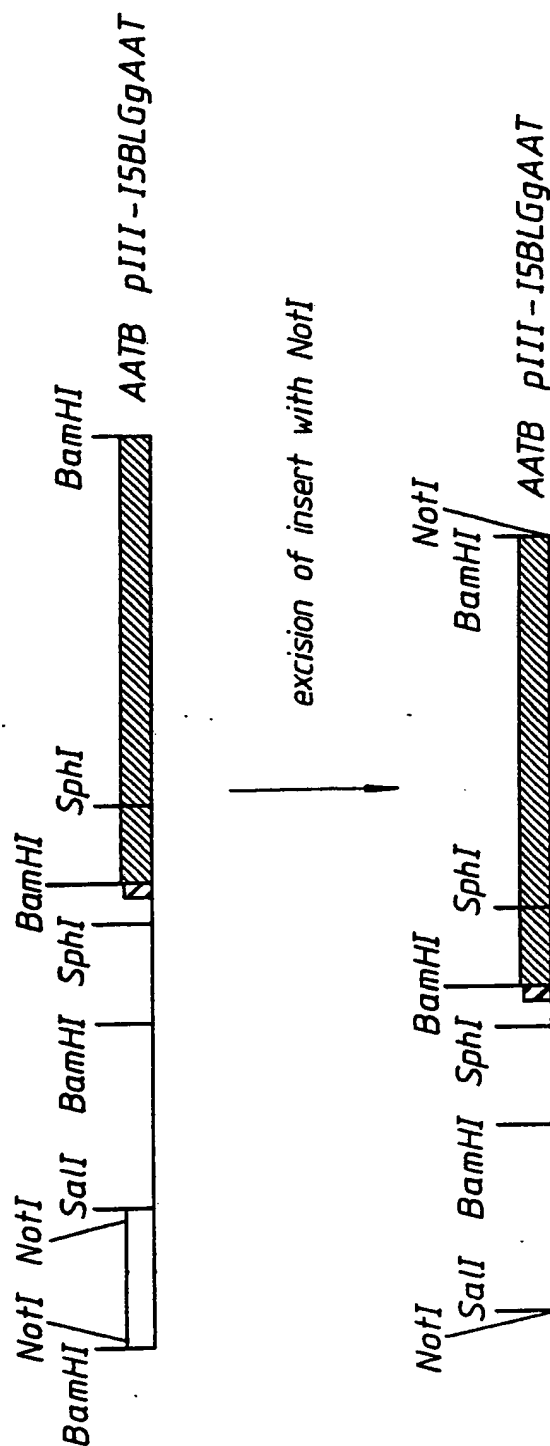


Fig.4.



5/21

SphI

gcatgcgcctcctgtataaggccccaagcctgctgtctcagccctcc

BLG|AAT

\*--&gt;

actccctgcagagctcagaagcagacccag|cgacaatgccgtcttct  
 PvuII-0 |TaqI-0 MetProSerSer

ValSerTrpGlyIleLeuLeuLeuAlaGlyLeuCysCysLeuValPro  
 gtctcgtggggcatcctcctgctggcaggcctgtgctgcctgggtccct  
 ^^^

BamHI

ValSerLeuAlaGluAspProGlnGlyAsp  
 gtctccctggctgaggatccccaggagat:

*Sequence of AATB (pIII-15BLGgAAT) from the SphI site corresponding to the 5' flanking sequences of  $\beta$ -lactoglobulin through the fusion to the  $\alpha$ 1-antitrypsin sequences. The key restriction sites for SphI and BamHI are underlined.*

\* = transcription start point

BLG =  $\beta$ -lactoglobulin

AAT =  $\alpha$ 1-antitrypsin

^^^ = indicate three nucleotides missing from the published sequence of Ciliberto, Dente & Cortese (1985)

Cell 41, 531-540, but clearly present in the clone

p8 $\alpha$ 1ppg procured from these authors. The nucleotides are present in the published sequence of  $\alpha$ 1-antitrypsin

described by Long, Chandra, Woo, Davie & Kurachi (1984)

Biochemistry 23, 4828-4837.

Fig. 5.

6/21

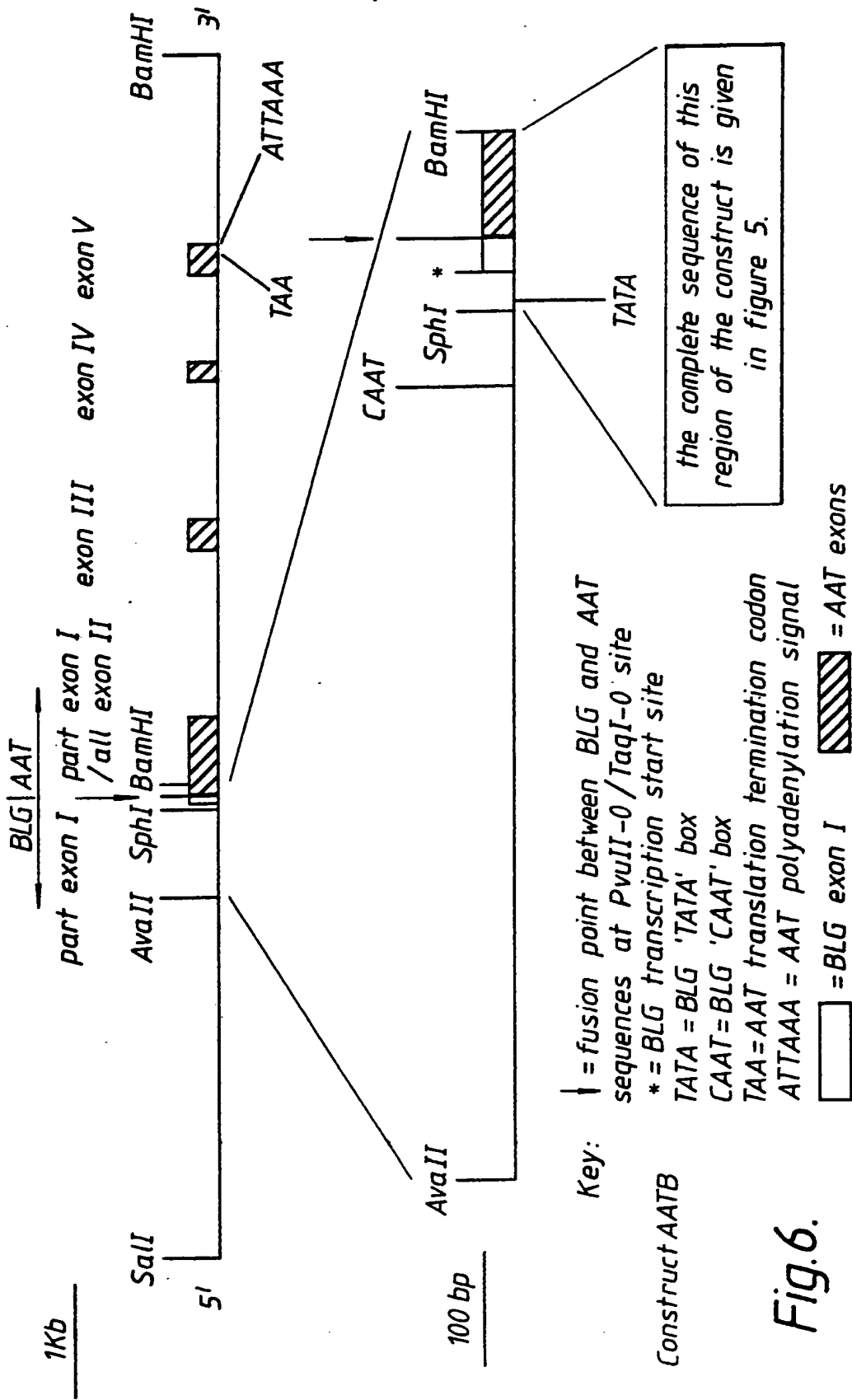


Fig.6.

7/21

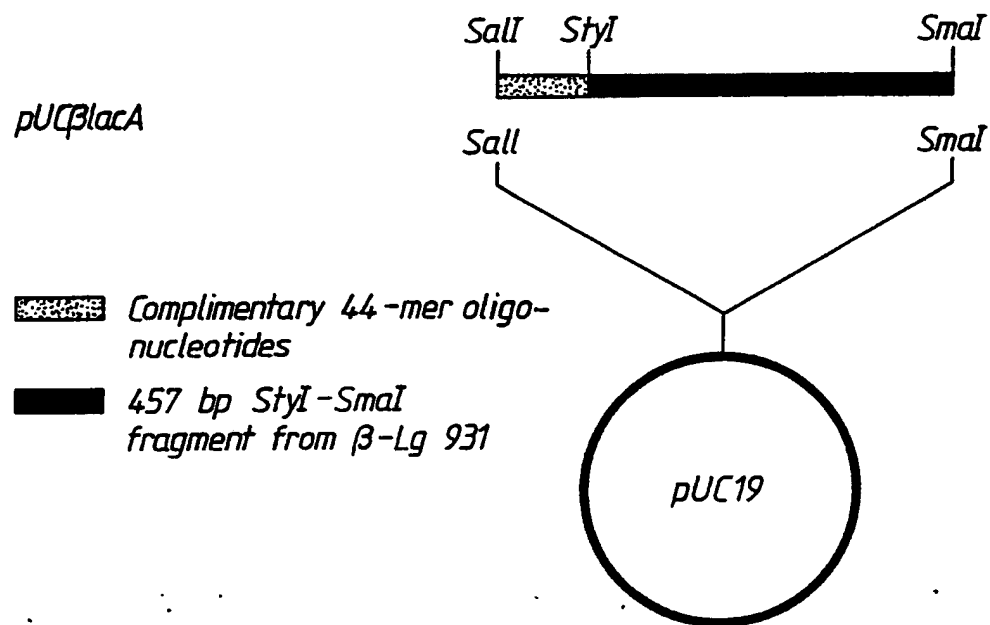
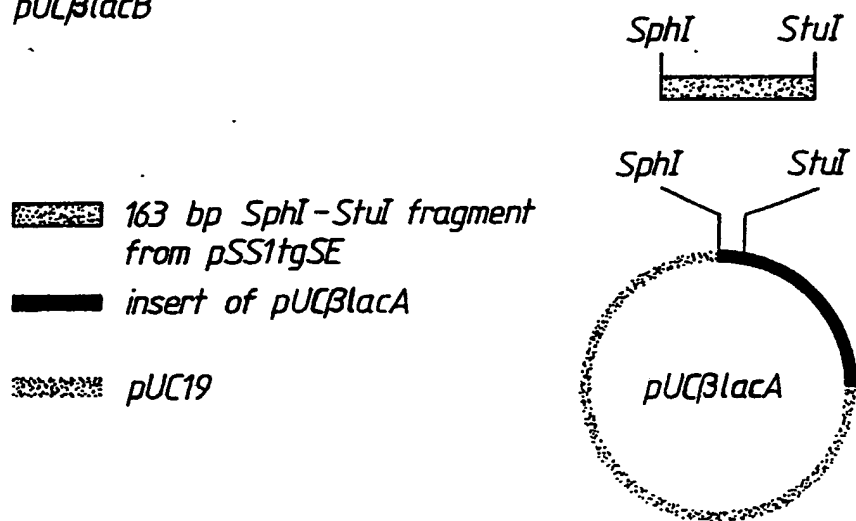
Construction of *pSS1tgXSΔClaBLG(BB)**pUCβlacB*

Fig. 7.

8/21


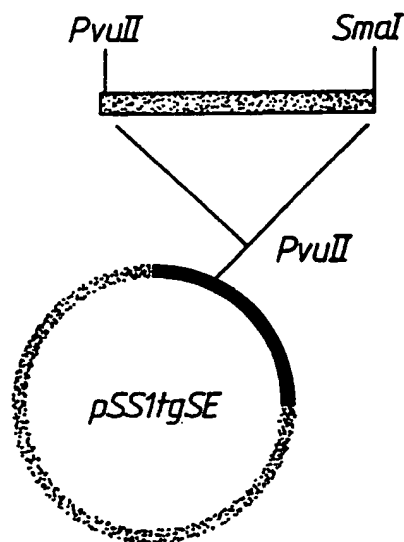

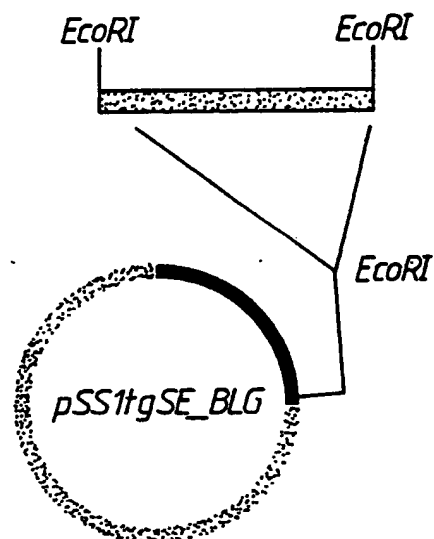

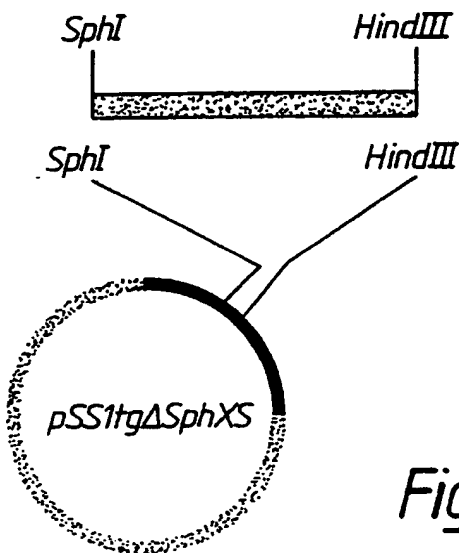
*pSS1tgSE\_BLG*
 *ppoly*
 insert of *pSS1tgSE*
*pSE\_BLG\_3'*
 5.3 *EcoRI* partial fragment from *pSS1tgXSΔCla*
 *ppoly*
 insert
*pSS1tgXSΔClaBLG*
 3 kb *SphI*-*HindIII* fragment from *pSE\_BLG\_3'*
 insert of *pSS1tgΔSphXS*
 *ppoly*


Fig. 8.

9/21

Construction of AATC:pSS1pUCXSAAT.TGA

1. Synthesis of oligonucleotides: 5' CTTGTGATATCG  
3' CACTATAGCTTAA 5'
2. Ligate annealed oligos into *StyI*/*EcoRI* cleaved *pSS1tgSE* to construct plasmid *pSS1tgSE.TGA*



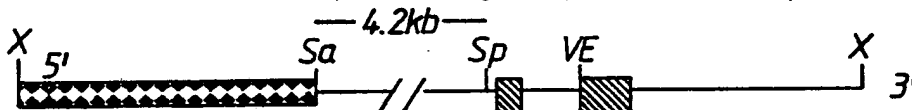
3. Cleave with *EcoRI*: Blunt with *Klenow* polymerase. Second cleavage with *SphI*. Isolate *SphI*-*EcoRI* (blunted) fragment.



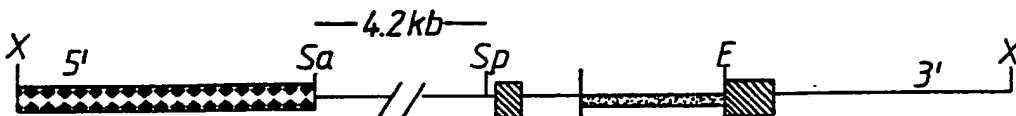
4. Cleave plasmid *pBJ7* (this patent) with *SphI* and *Pvu II*. Isolate large 4.3 kb) fragment.
5. Ligate *SphI*-*EcoRI*(blunt) fragment (3) with *SphI*-*PvuII* fragment (4) to produce *pSS1tgSpX.TGA*



6. Isolate *SphI*-*XbaI* insert from *pSS1tgSpX.TGA* (5) and ligate to 4.2 kb *SalI*-*SphI* fragment from *pSS1tgXS* (previous patent) and *XbaI*-*SalI* cleaved *pUC18* to yield *pSS1pUCXS.TGA*



7. Insert *AccI*-*HindIII* AAT insert from *pUC8a1AT.73* (this patent) into the unique *EcoRV* site of *pSS1pUCXS.TGA* to produce *pSS1pUCXSAAT.TGA*. For microinjection the *XbaI*-*SalI* fragment is excised from the vector.



*pPOLY*; 
 *pUC18*; 
 — *BLG* intron or flanking,

*BLG* exons, 
 *AAT*, 
 | *oligo*.

*E*, *EcoRI*; *X*, *XbaI*; *Sa*, *SalI*; *Sp*, *SphI*; *V*, *EcoRV*; *St*, *StyI*; *P(0)*, inactivated *PvuII* site.

Fig. 9.

10/21

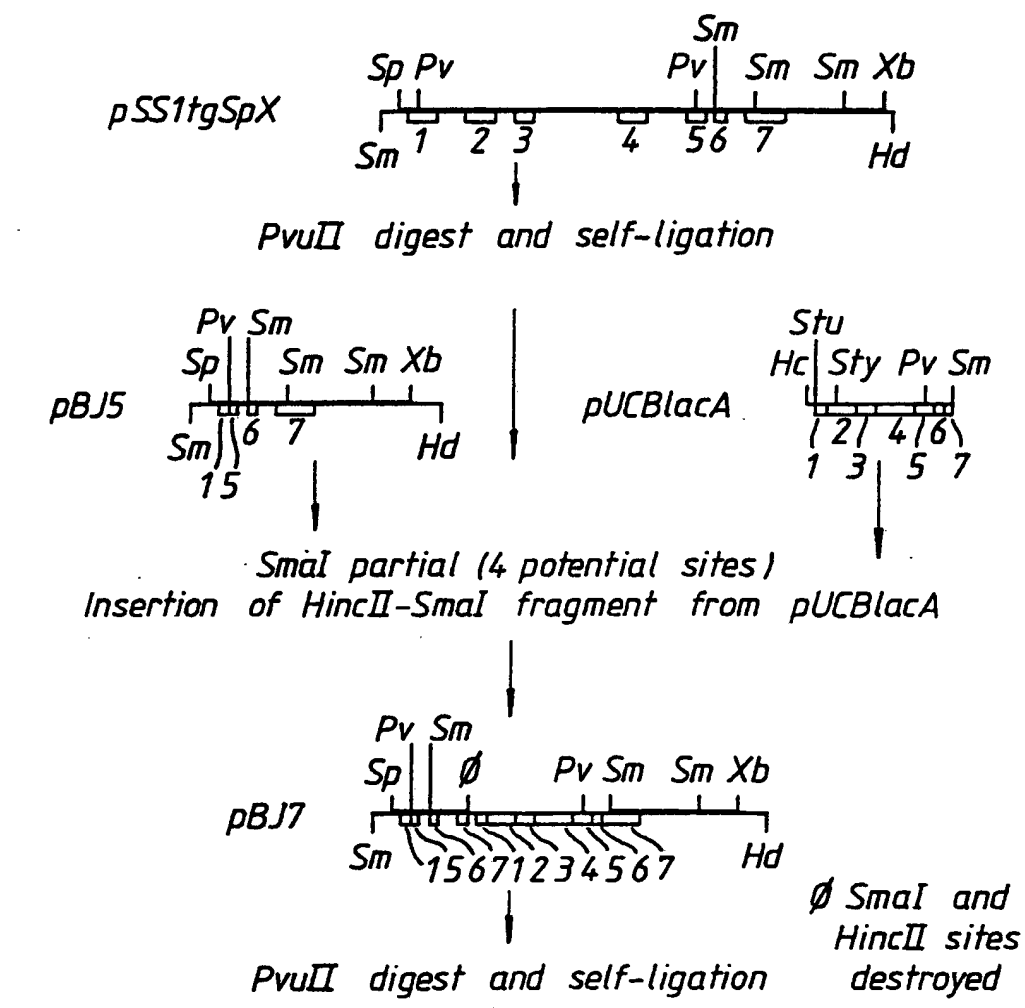


Fig.10a.

11/21

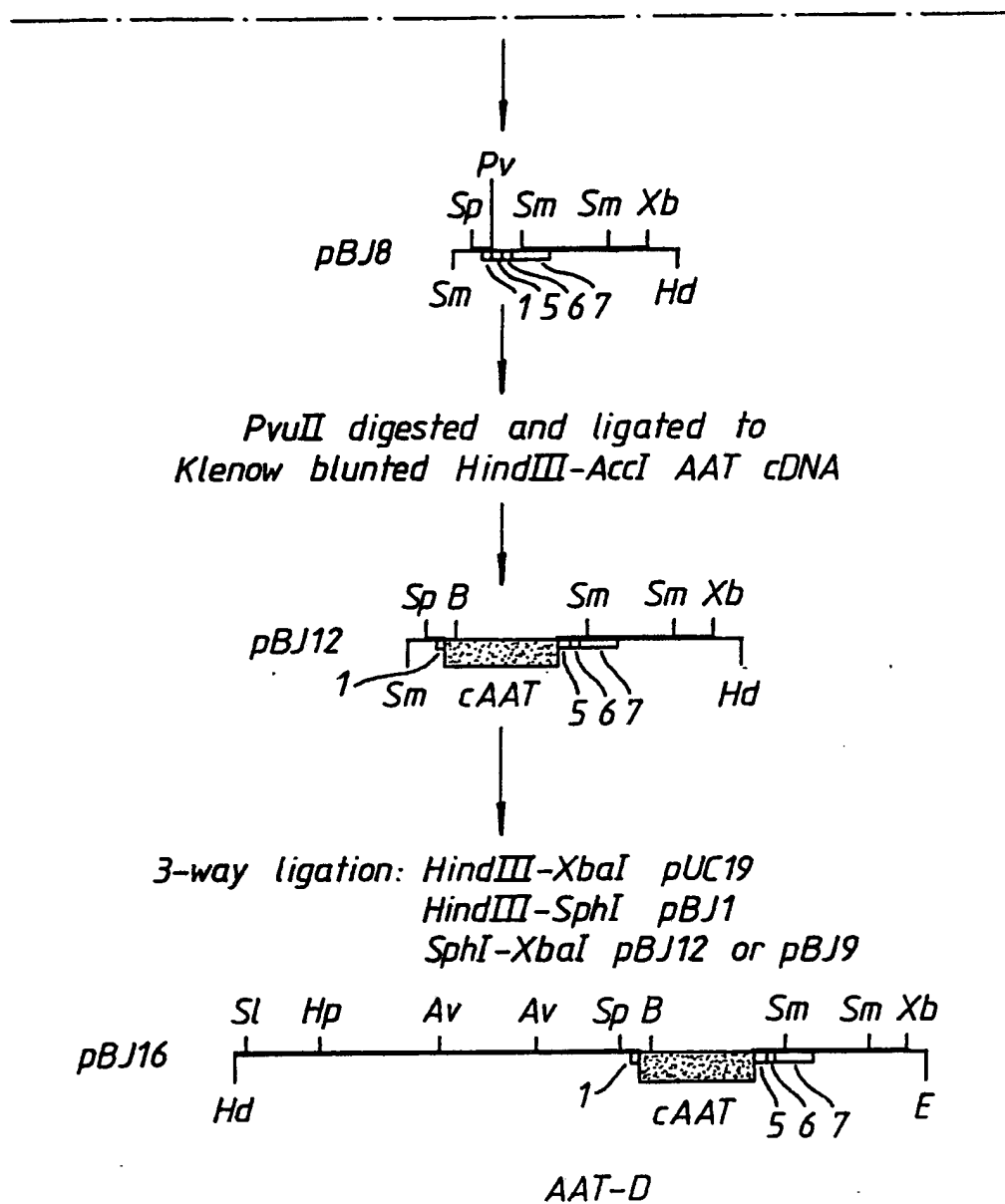
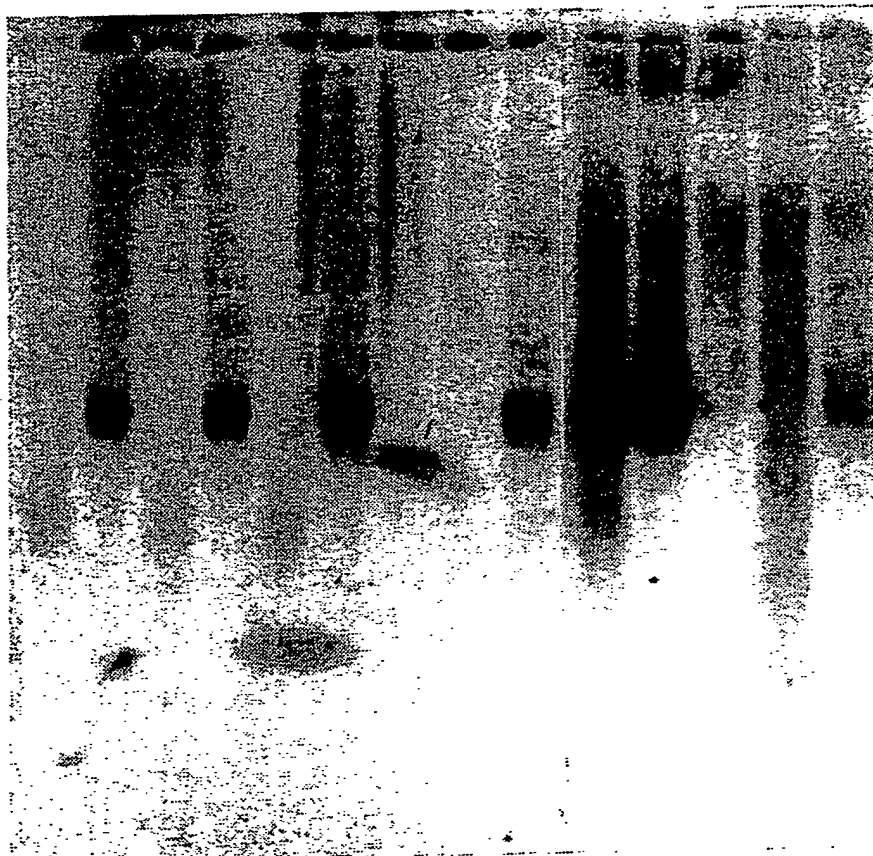


Fig.10b.

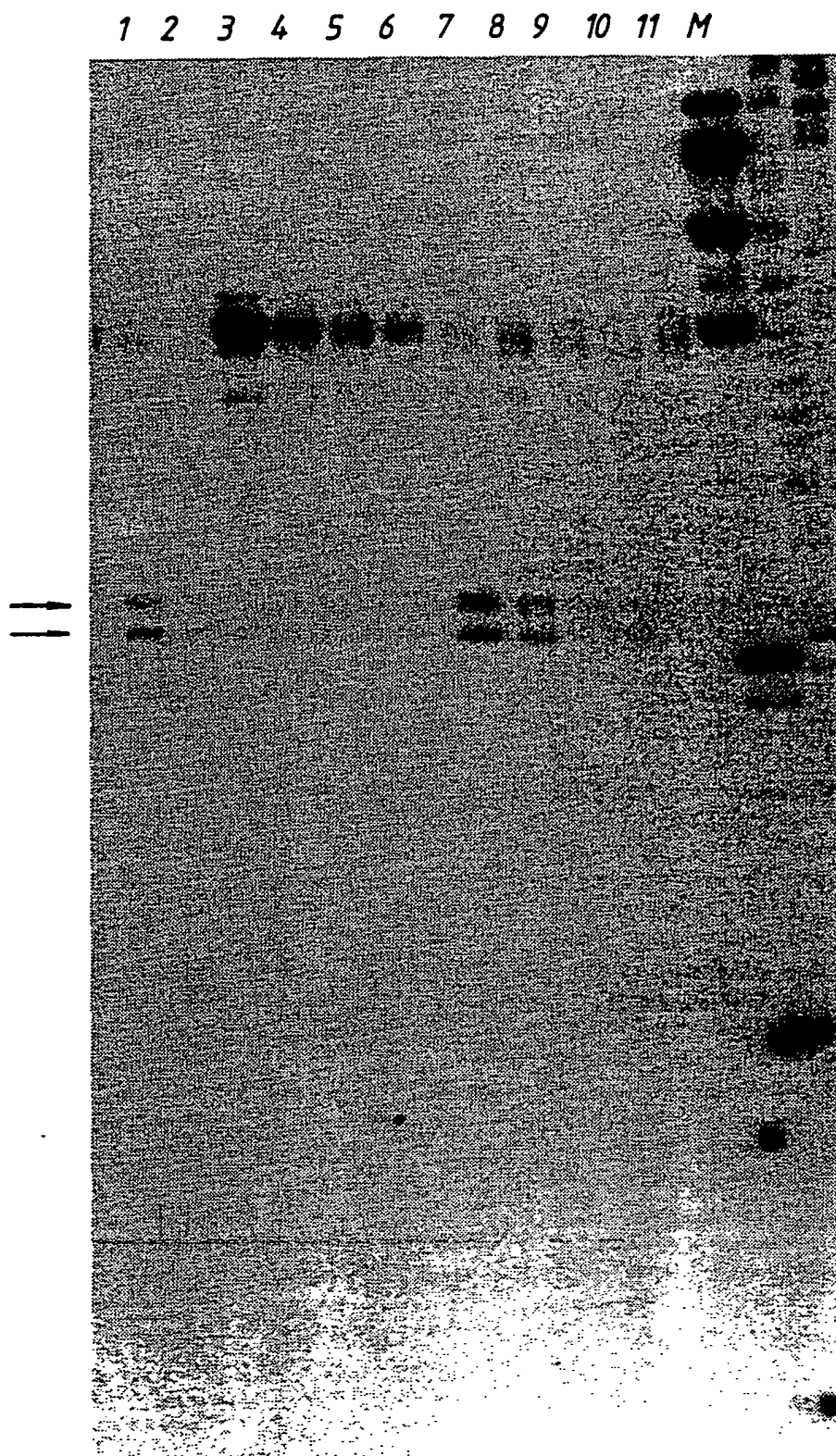
12/21

1 2 3 4 5 6 7 8 9 10 11 12 13 14  
M L M L M L K Sp Sa M L K Sp Sa

*Fig.11.*



13/21

*Fig.12.*

SUBSTITUTE SHEET

14/21

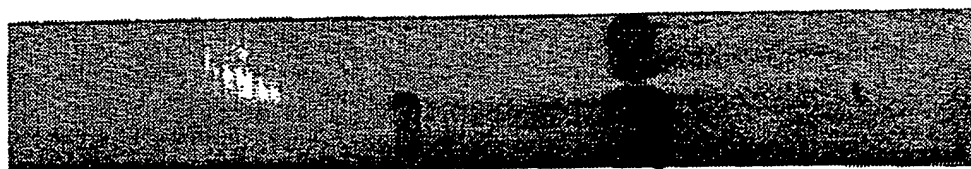


Fig.13.

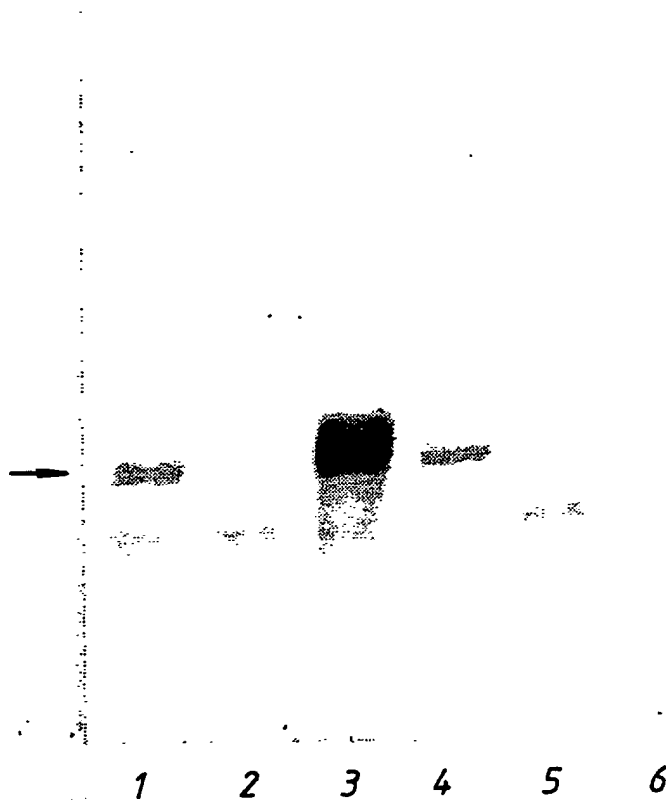
8 9



1 2 3 4 5 6 7

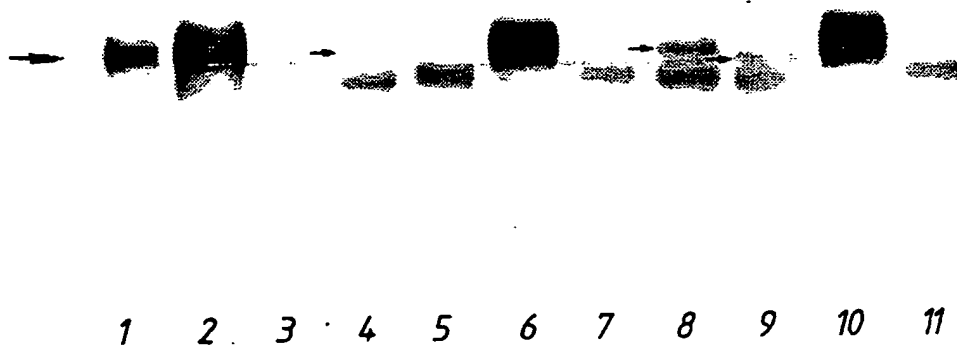
15/21

*EXPRESSION OF HUMAN AAT IN TRANSGENIC SHEEP MILK*



*Fig.14.*

*EXPRESSION OF HUMAN AAT IN THE MILK OF TRANSGENIC MICE*



*Fig.15.*

16/21

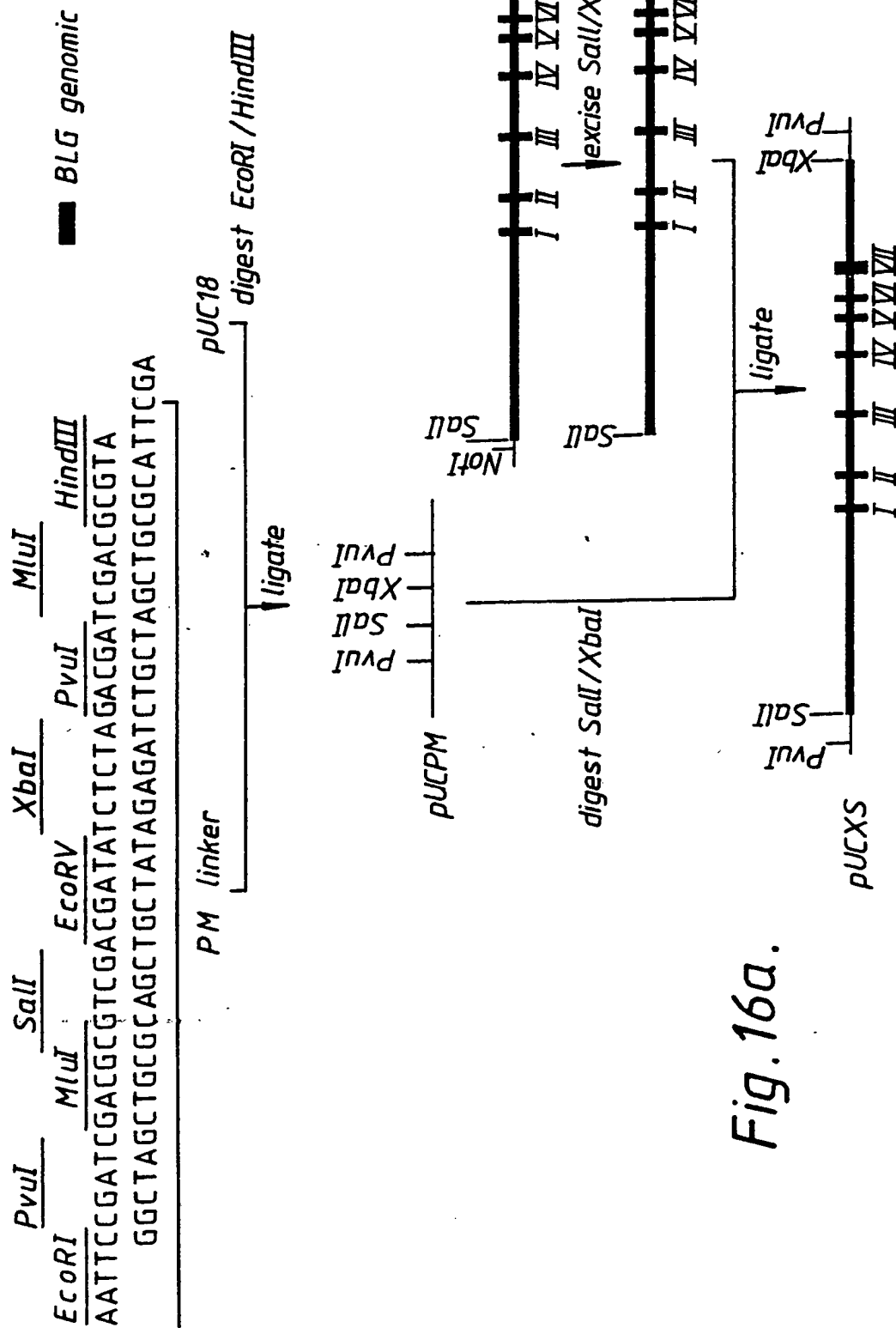


Fig. 16a.

17/21

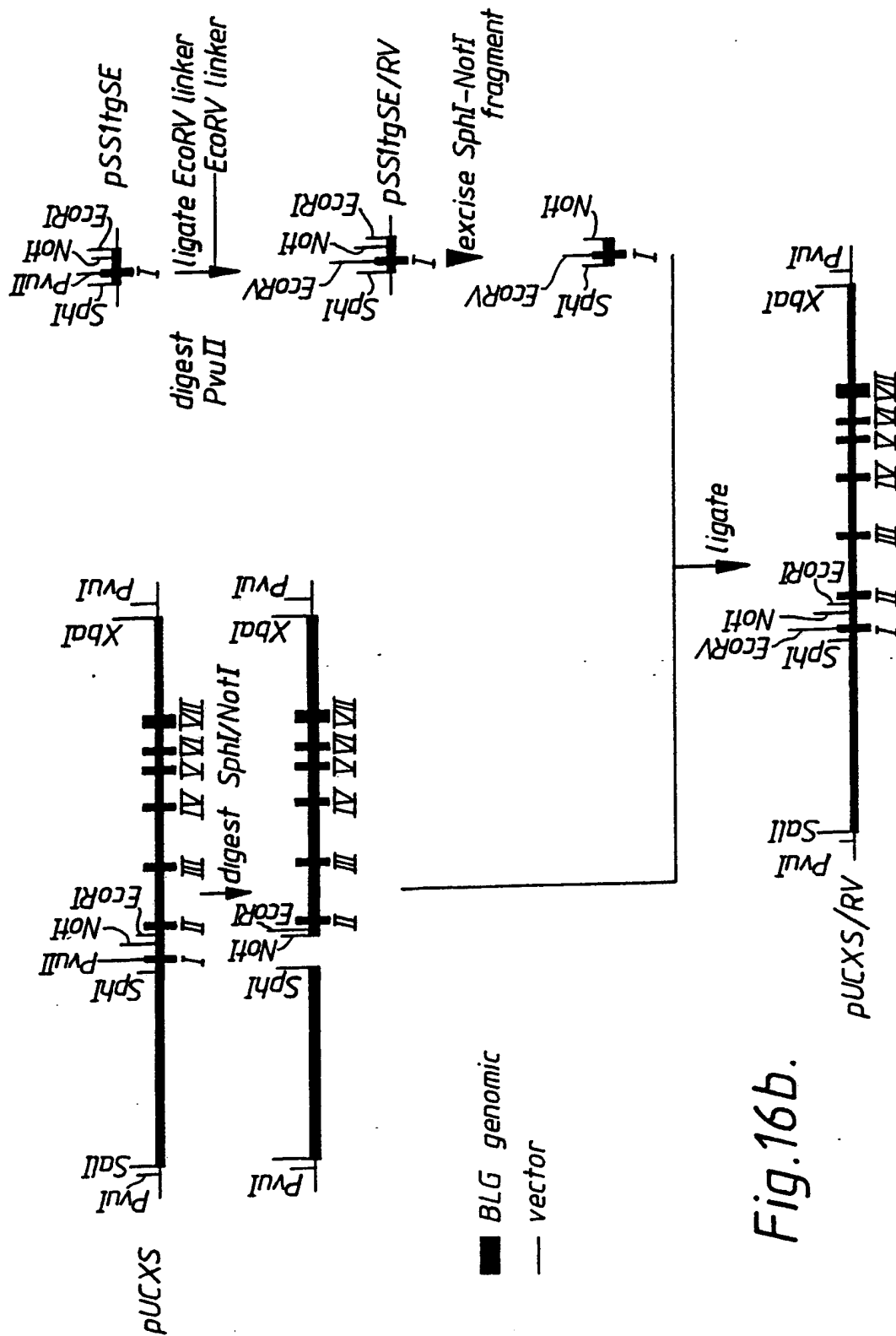
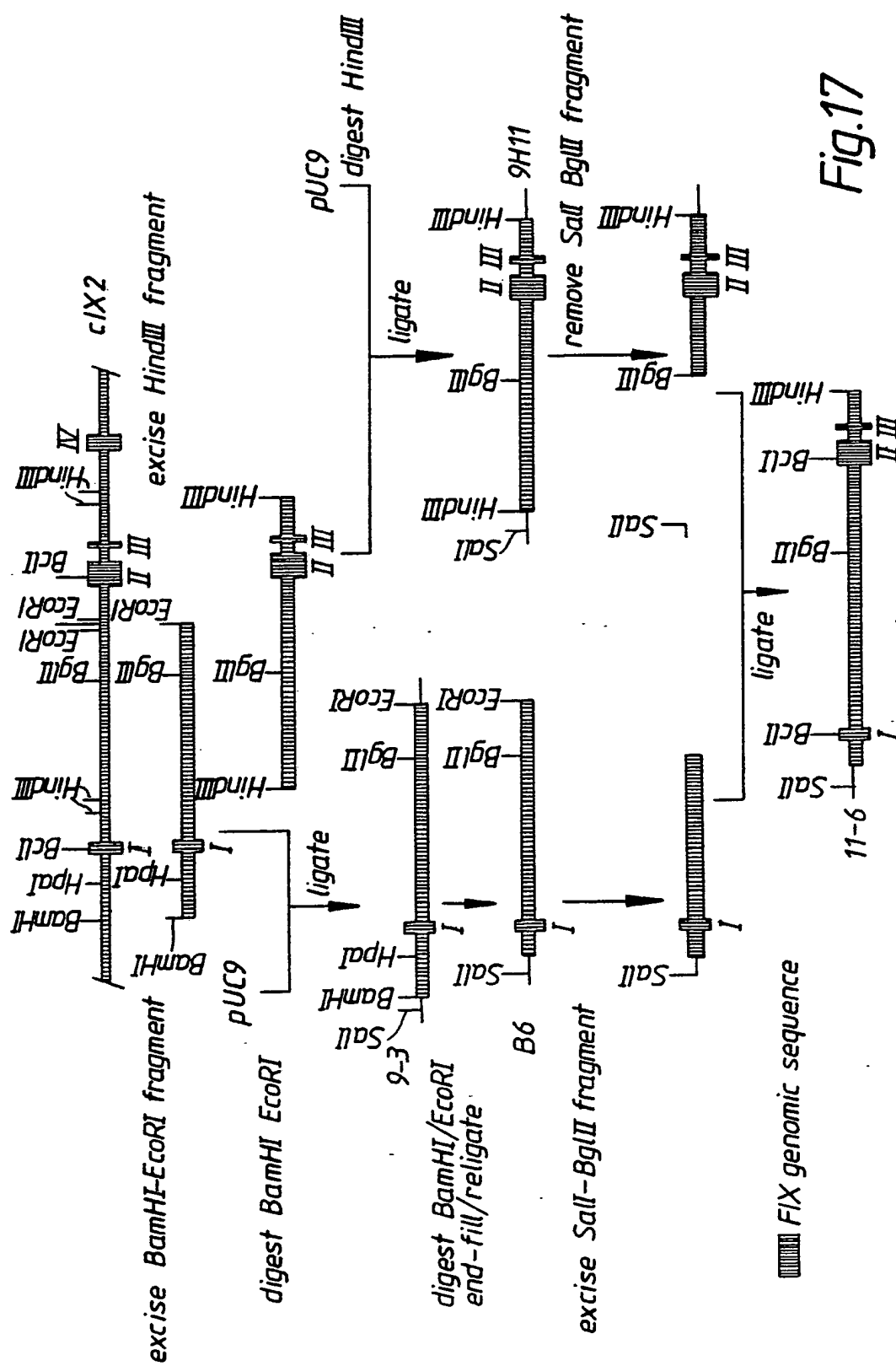


Fig.16b.

18/21



19/21

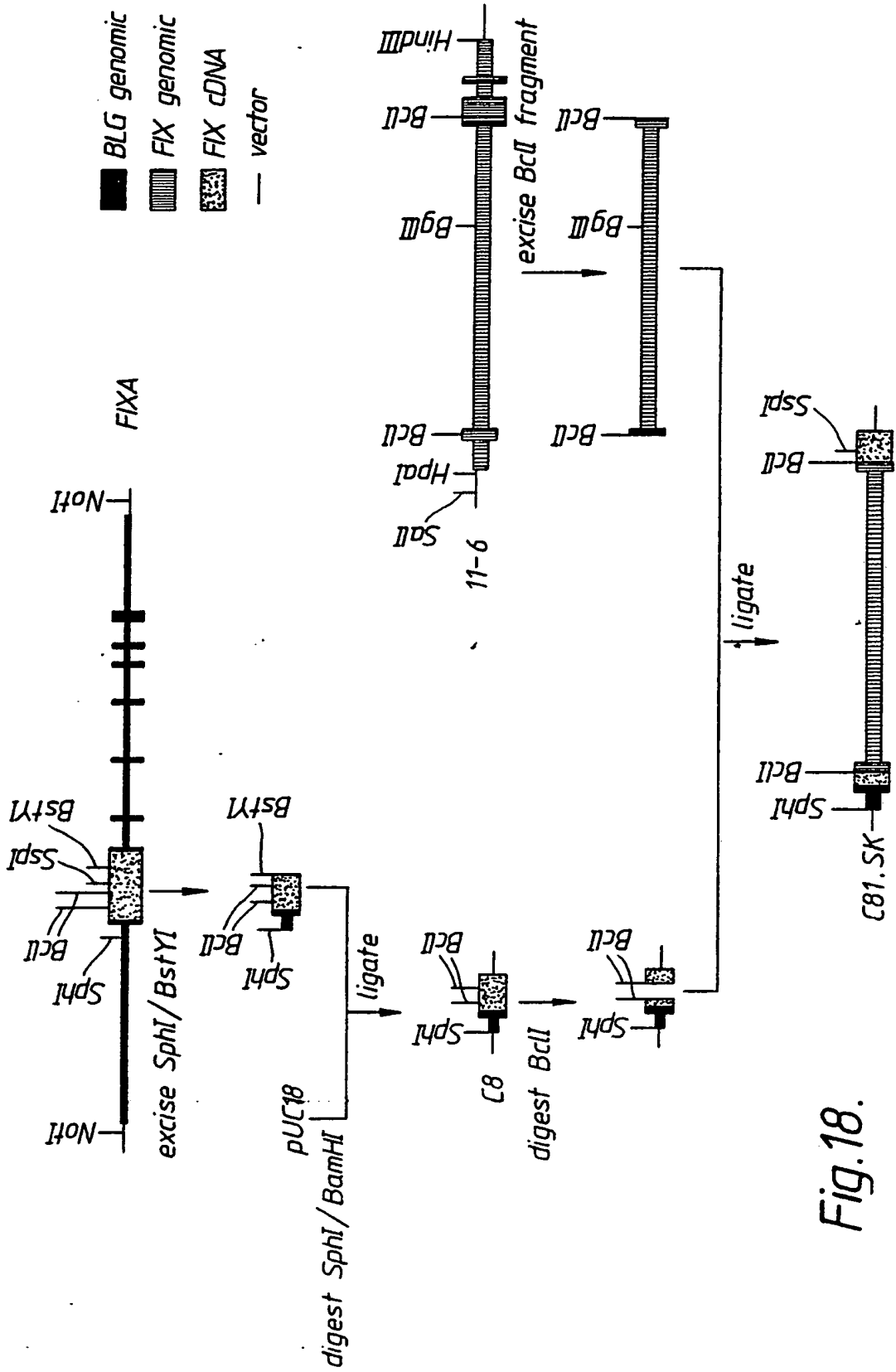


Fig.18.

20/21

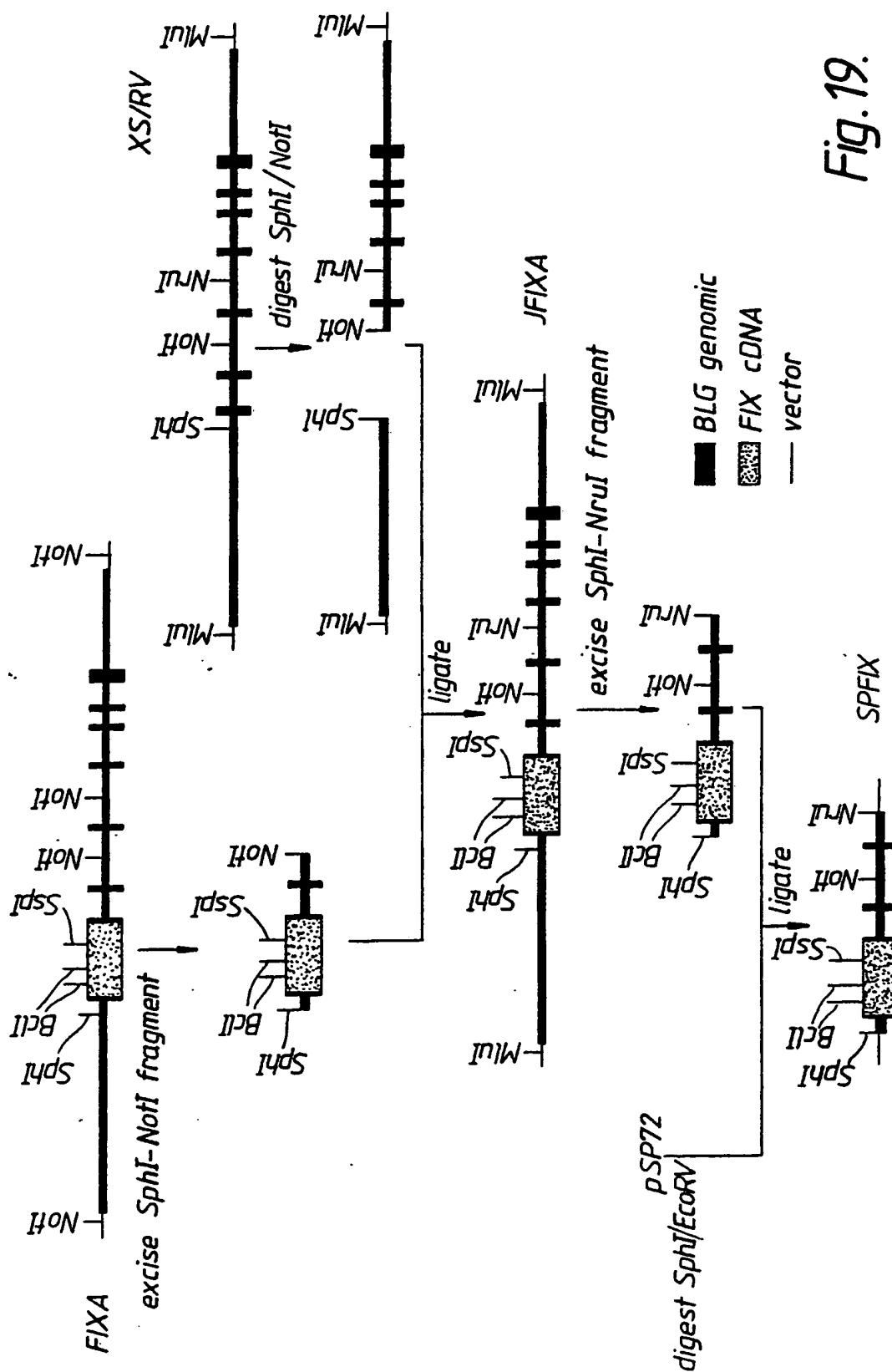


Fig. 19.



21/21

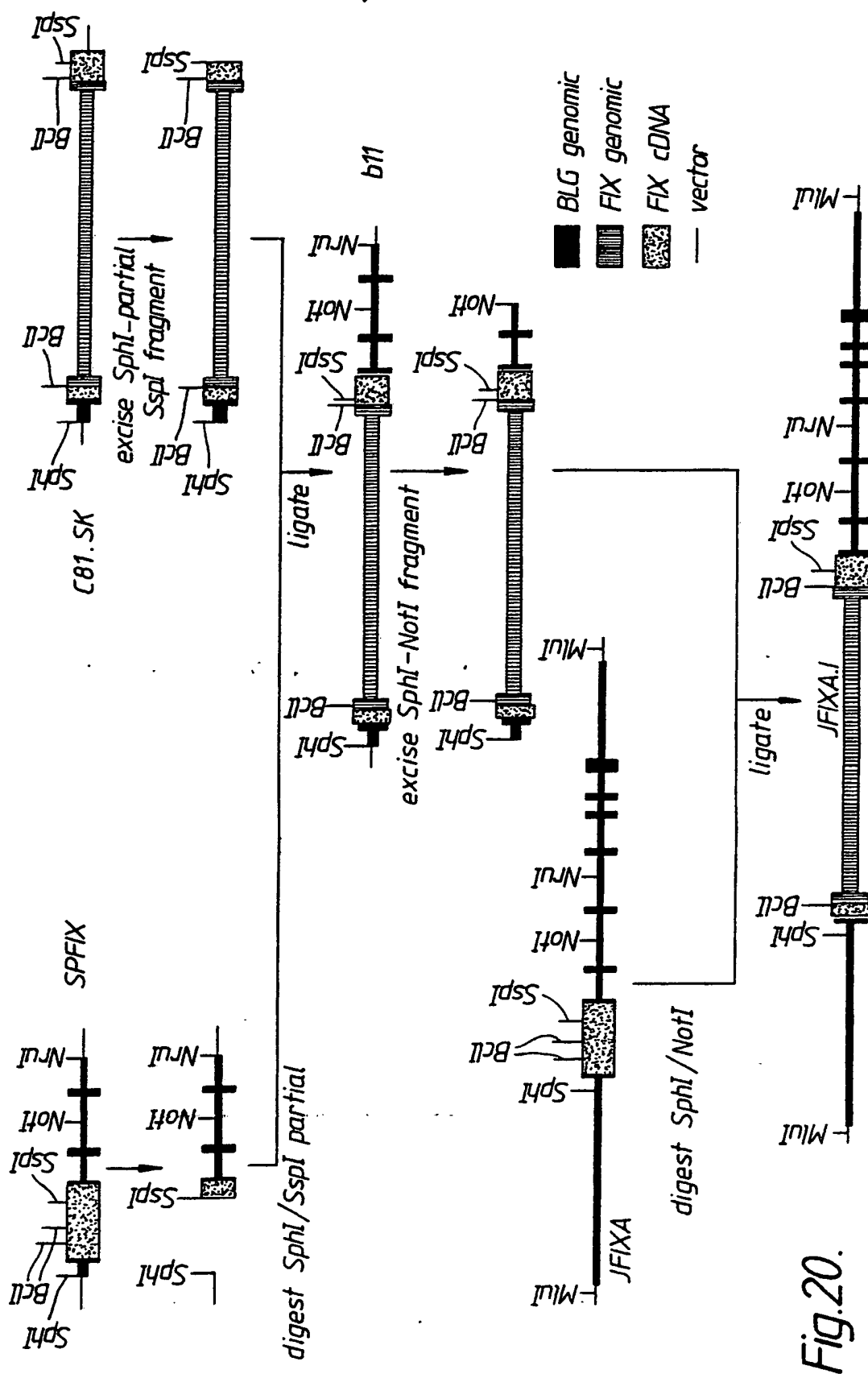


Fig.20.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01343

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 15/85, C 12 N 15/57														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <div style="display: flex; justify-content: space-between; border-bottom: 1px solid black; margin: 5px 0;"> <span>Classification System :</span> <span>Classification Symbols</span> </div> <div style="display: flex; justify-content: space-between; margin: 5px 0;"> <span>IPC5</span> <span>C 12 N</span> </div> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched <sup>8</sup></div>														
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <thead> <tr> <th style="width: 10%;">Category <sup>9</sup></th> <th style="width: 70%;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Proc.Natl.Acad.Sci., Vol. 85, 1988,(USA) Ralph L. Brinster et al: "Introns increase transcriptional efficiency in transgenic mice", see page 836 - page 840  --</td> <td style="text-align: center; vertical-align: top;">1-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">WO, A1, 88/00239 (PHARMACEUTICAL PROTEINS LTD) 14 January 1988, see page 19, line 10 - line 20; claim 20  --</td> <td style="text-align: center; vertical-align: top;">1-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">EP, A1, 0264166 (INTEGRATED GENETICS, INC.) 20 April 1988, see the whole document  --</td> <td style="text-align: center; vertical-align: top;">1-17</td> </tr> </tbody> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	Proc.Natl.Acad.Sci., Vol. 85, 1988,(USA) Ralph L. Brinster et al: "Introns increase transcriptional efficiency in transgenic mice", see page 836 - page 840  --	1-17	Y	WO, A1, 88/00239 (PHARMACEUTICAL PROTEINS LTD) 14 January 1988, see page 19, line 10 - line 20; claim 20  --	1-17	Y	EP, A1, 0264166 (INTEGRATED GENETICS, INC.) 20 April 1988, see the whole document  --	1-17
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>												
Y	Proc.Natl.Acad.Sci., Vol. 85, 1988,(USA) Ralph L. Brinster et al: "Introns increase transcriptional efficiency in transgenic mice", see page 836 - page 840  --	1-17												
Y	WO, A1, 88/00239 (PHARMACEUTICAL PROTEINS LTD) 14 January 1988, see page 19, line 10 - line 20; claim 20  --	1-17												
Y	EP, A1, 0264166 (INTEGRATED GENETICS, INC.) 20 April 1988, see the whole document  --	1-17												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search  <b>24th January 1990</b> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center;">JAN 25 1990</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;">           International Searching Authority  <div style="text-align: center;"><b>EUROPEAN PATENT OFFICE</b></div> </td> <td style="width: 50%; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;"> <b>T.K. WILLIS</b> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <b>24th January 1990</b>	Date of Mailing of this International Search Report <div style="text-align: center;">JAN 25 1990</div>	International Searching Authority <div style="text-align: center;"><b>EUROPEAN PATENT OFFICE</b></div>	Signature of Authorized Officer <div style="text-align: center;"> <b>T.K. WILLIS</b> </div>								
Date of the Actual Completion of the International Search <b>24th January 1990</b>	Date of Mailing of this International Search Report <div style="text-align: center;">JAN 25 1990</div>													
International Searching Authority <div style="text-align: center;"><b>EUROPEAN PATENT OFFICE</b></div>	Signature of Authorized Officer <div style="text-align: center;"> <b>T.K. WILLIS</b> </div>													

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,A	Chemical Abstracts, volume 110, no. 19, 8 May 1989, (Columbus, Ohio, US), Deng, Tiliang et al.: "Thymidylate synthase gene expression is stimulated by some (but not all) introns", see page 199, abstract 167168n, & Nucleic Acids Res 1989, 17 (2), 645- 58  -----	1
-----	--	---

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 15, 16 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(ii)

Plant or animal varieties or essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.

2. ☐ Claim numbers ..... because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/01343**

SA 32133

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office E.I.P. file on 08/11/89  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 88/00239	14/01/88	AU-D- 76490/87	29/01/88
		EP-A- 0274489	20/07/88
		JP-T- 1500162	26/01/89
EP-A1- 0264166	20/04/88	JP-A- 63000291	05/01/88

1 a hundred animals or lines express. This is  
2 particularly the case for large animals, for which,  
3 with the techniques currently available, much time and  
4 money can be expended to produce only a small number of  
5 GO animals.

6  
7 Early work with transgenic animals, as represented by  
8 WO-A-8800239 has used genetic constructs based on cDNA  
9 coding for the protein of interest. The cDNA will be  
10 smaller than the natural gene, assuming that the  
11 natural gene has introns, and for that reason is more  
12 easy to manipulate.

13  
14 Brinster et al (PNAS 85 836-840 (1988)) have  
15 demonstrated that introns increase the transcriptional  
16 efficiency of transgenes in transgenic mice. Brinster  
17 et al show that all the exons and introns of a natural  
18 gene are important both for efficient and for reliable  
19 expression (that is to say, both the levels of the  
20 expression and the proportion of expressing animals)  
21 and is due to the presence of the natural introns in  
22 that gene. It is known that in some cases this is not  
23 attributable to the presence of tissue-specific  
24 regulatory sequences in introns, because the phenomenon  
25 is observed when the expression of a gene is redirected  
26 by a heterologous promoter to a tissue in which it is  
27 not normally expressed. Brinster et al say that the  
28 effect is peculiar to transgenic animals and is not  
29 seen in cell lines.

30  
31 It might therefore be expected that the way to solve  
32 the problems of yield and reliability of expression  
33 would be simply to follow the teaching of Brinster et